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Antimicrobial Activity of Cinnamaldehyde or Geraniol alone or Combined with High Pressure Processing to Destroy Escherichia coli O157:H7 and Salmonella enterica in Juices

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Antimicrobial activity of cinnamaldehyde or geraniol alone or combined with high pressure processing to destroy *escherichia coli* O157:H7 and *salmonella enterica* in juices

by

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CHAPTER 1. GENERAL INTRODUCTION

Introduction

Bacterial pathogens causing foodborne diseases and outbreaks are of public health significance. The Centers for Disease Control and Prevention estimate that each year approximately 48 million Americans (1 in 6 individuals) acquire a foodborne illness, over 127,000 are hospitalized, and 3,000 die. A majority of the illnesses, hospitalizations and deaths that occur each year are attributed to the following pathogens: norovirus (2.2 million), *Salmonella* (3.6 million), *Clostridium perfringens* (342k), *Campylobacter spp.* (1.9 million), *Staphylococcus aureus*, *Toxoplasma gondii*, *E. coli* O157:H7, and *Listeria monocytogenes* (CDC, 2014a). The economic impact associated with those pathogens in the United States is substantial. The approximate annual economic burden as a result of those pathogens ranges from $342,000 to $9.4 million (Hoffman, 2015). It is nearly impossible to fully eliminate bacterial pathogens; therefore, it is imperative that the government, processors, food manufacturers, and retailers make efforts to ensure that the incidence of foodborne pathogens is reduced consumer safety.

As more manufacturers formulated products with synthetic preservatives, consumers have raised the issue of potential negative health effects and have thus created a demand for naturally derived food additives and minimally processed foods (White, 2011). This consumer driven effort to push food manufacturers to replace chemical or synthetic ingredients with more natural ones is known as green consumerism. Sorbates, nitrites, sulfites, and parabens are common examples of "chemical" preservatives. The biggest concern from consumers is the potential long term adverse health effects that these chemicals might have on human health. Antimicrobials that are considered "green" should possess the following characteristics: 1) low mammalian toxicity/cytotoxicity, 2) low skin sensitization potential, 3) low eco-toxicity, 4) low bioaccumulation potential, 5) low

emission potential during manufacturing, application or post application, 6) no or low volatile organic compound (VOC) content, as applicable, and 7) complete effectiveness against the relevant target organisms (Sofos, 1998; Kruger and Mann, 2003; Shrankel, 2004; Davidson et al., 2005; Rulis, 2009; White, 2011). There is high potential for natural antimicrobials to be used in preserving foods, extending shelf life and vastly improving overall food quality, all while promoting this image of "green" products. Before the food industry can fully transition to using only natural ingredients, research must be conducted to determine overall costs, limits of antimicrobial activity, optimal concentration, applicability of hurdle technology, and federal regulations, if any, regarding food applications (White, 2011).

The food industry is addressing green consumerism by gradually developing antimicrobials extracted from plant, animal and microbial sources for use in foods. Essential oils are substances that contain various aromas, flavors, and antimicrobial compounds isolated from plants or plant material. Two such essential oils of interest are cinnamaldehyde and geraniol. Cinnamaldehyde, a phenylpropene aldehyde, is isolated from cinnamon, and is a potential candidate for use as a natural antimicrobial due to it activity against both Gram negative and Gram positive bacteria. Geraniol is an alcohol isolated from sources such as citronella oil and palmarosa oil and is characterized as having a floral aroma. Some antimicrobials may lack the ability to inhibit bacterial pathogens at low concentrations and therefore may need to be used in combination with a process intervention (hurdle concept). A potential process intervention to be used with natural antimicrobial is high pressure processing. High pressure processing is an emerging technology that has demonstrated the ability to reduce bacterial populations in foods while maintaining the desirable attributes of the food. Combining high pressure processing and natural antimicrobials can possibly increase the efficacy of the antimicrobial systems far better than if each intervention were used alone against

foodborne pathogens. Thus, the first objective of this research was to establish the minimum inhibitory concentration of cinnamaldehyde and geraniol against gram negative bacteria in laboratory broth. The second objective was to evaluate the ability of cinnamaldehyde and geraniol to eliminate gram negative pathogens in carrot and mixed berry juice. The third objective was to evaluate the effectiveness of combining high pressure processing and cinnamaldehyde to destroy gram negative pathogens in both carrot juice and mixed berry juice. The fourth objective was to attempt to determine how cinnamaldehyde might be exerting its bactericidal action against *Escherichia coli* O157:H7.

Dissertation Organization

This dissertation is divided into seven chapters. The first chapter is comprised of a general introduction. The second chapter is a literature review containing information related to the research conducted in chapters 3 through 6. The final chapter provides a general summary of the research performed. All pertinent figures, tables, and graphs appear at the end of their respective chapters, which all follow a specified journal format. At the conclusion of each chapter references can be found formatted for journal specifications. It is the intent that Chapter 3 be submitted to Foodborne Pathogens and disease, and Chapters 4, 5, and 6 to Journal of Food Protection. The abstract titled "Antibacterial effectiveness of cinnamaldehyde against *Escherichia coli* O157:H7 and *Salmonella enterica* in carrot and blackberry juice blends held at 4 °C" was presented at the annual meeting of the International Association of Food Protection in Portland Oregon (July 2015).

CHAPTER 2. LITERATURE REVIEW

Foodborne outbreaks in juice

Fruit and vegetable juices are increasingly being purchased by consumers due to the perceived health benefits that they provide. Although safety measures are in place for production of fruit and vegetable juices, incidents of foodborne outbreaks in juices have risen. Over the past several decades, juices have been implicated in several foodborne outbreaks. Between 1995 and 2005, 21 outbreaks associated with juices were reported in the United States (Table 1.) (Vojdani et al., 2008; Bates et al., 2001). The main pathogens responsible for the outbreaks were *Salmonella*, *Escherichia coli* O157:H7, and *Cryptosporidum* and those were most often implicated in orange juice, apple cider, and apple juice (Bates el al., 2001). Most cases of outbreaks were attributed to the consumption of unpasteurized juices (Vojdani et al., 2008). In 1998, in an attempt to lower the occurrence of outbreaks in ciders, legislation requiring all unpasteurized juices to be labeled as raw was passed (FDA, 1998). To further strengthen food safety practices in the manufacturing of juice, legislation was passed requiring all juice processors to create/implement a Hazard Analysis and Critical Control Point (HACCP) plan as well as requiring a cumulative 5-Log_{10} CFU/ml reduction in the relevant pathogen (FDA, 2001). An example of a relevant pathogen is *Escherichia coli* O157:H7 found in apple juice or *Salmonella enterica* in orange juice.

Table 1. Fruit juice associated outbreaks from 1995-2000. (Source: Adapted from Bates et al., 2001).

Juice Product (Year)	**Infectious Agent**
Orange juice (1995)	*S. hartford, S. gaminera, S. rubislaw*
Apple juice (1996)	*E. coli* O157:H7
Apple juice (1996)	*E. coli* O157:H7
Apple juice (1996)	*Cryptosporidium parvum*
Apple cider (1997)	*E. coli* O157:H7
Orange juice (1998)	*Salmonella*
Apple cider (1998)	*E. coli* O157:H7
Mamey juice (1999)	*Salmonella typhi*
Orange juice (1999)	*Salmonella enterica*
Orange juice (1999)	*Salmonella muenchen*
Apple cider (1999)	*E. coli* O157:H7
Orange, grapefruit, lemonade (2000)	*Salmonella enterica*

Current and Emerging Juice Processing Methods

Pasteurization

Pasteurization was named after Louis Pasteur, the scientist credited with discovering the lethality of heat on microorganisms and the use of thermal treatment to preserve food. Pasteurization of juice is aimed at: 1) inactivation of the natural enzymes to avoid juice quality deterioration, 2) stabilization of juice characteristics such as color, turbidity and aroma, 3) reduction of the microbial load of the juice to a very low concentration, 4) avoidance of juice spoilage, and 5) the conservation of the juice in good condition during its commercial life (Falguera et al., 2014; Tressler and Joslyn 1961; Nagy et al. 1977, 1992). The level of pasteurization required is dependent on the following factors: pH (low pH is inhibitory to many microorganisms), water activity (higher available free water results in increased microbial growth), oxygen (presence of oxygen in combination with nutrients and water allow for microorganism growth), microbial quality of raw material (an initial concentration of microorganisms found naturally on raw foods

enter the processing facility via the fruit, transport containers, or product handlers), and rheological properties of the juice (the product's viscosity, °Brix, etc will determine the ability for heat to transfer) (Falguera et al., 2014; Nagy et al., 1977).

Ohmic Heating

Ohmic heating is an alternative processing method that utilizes an electric current to eliminate microorganisms. This method allows for rapid and uniform heating of a product to reduce the microbial load, while minimizing thermal damage in comparison to other heat transfer methods (Falguera et al., 2014; Sarang et al., 2008). In a study conducted by Leizerson and Shimoni (2005), no significant differences were observed between conventional pasteurization and ohmic heating in regards to degradation of vitamin C or pectin esterase. It was also observed that both treatments prevented microbial growth for 105 days and the sensory life the juice treated by ohmic heating was twice as long as the conventionally pasteurized juice. Ohmic heating is a promising technology; however, further investigation is warranted to determine optimal parameters for improving shelf life and overall quality of juices.

Ultraviolet light

Ultraviolet light (UV) has wavelengths ranging from 10 to 400 nm on the electromagnetic spectrum. Most UV light emitted from the sun does not reach earth. The radiation that does reach earth is closest to visible light (300-400 nm) and is called near-UV radiation and contains less energy. Extreme-UV radiation (10-120nm) contains the highest energy content and a region known as far-UV radiation (120-200 nm) can break covalent bonds in a process called photoionization and thereby generate ionized compounds (NASA Science Mission Directorate 2011; World Health Organization 2013). Ionized compounds are quite unstable and will typically start a chemical

reaction with a nearby compound. Several compounds, such as DNA, are quite susceptible to UV light. In the case of DNA, when exposed to UV light, replication or DNA transcription is halted (Matsumura and Ananthaswamy 2002). Applications targeting microorganisms for destruction should determine the appropriate wavelength of UV light to use in addition to the exposure time in an effort to increase damage and overwhelm repair systems resulting in cellular death (Falguera et al., 2014). Although increasing exposure time may result in cell death, there is the potential for reduction in overall quality of the product. Investigators have reported on the effects of UV light on rheological properties (Shamsudina et al. 2013), induced off-flavors (Demirci and Krishnamurthy 2011), discolorations (Cuvelier and Berset 2005), and vitamin C loss (Falguera et al. 2012) in juice. Further studies should be conducted to further understand the full effects of UV light on juice.

Pulsed light

Pulsed light utilizes intense pulses of short duration and broad spectrum radiation to decontaminate food as well as food-contact materials. UV, infrared, and white light comprise the broad spectrum radiation. Oms-Oliu et al. (2010) have suggested responses to photochemical and also photothermal effects induced by the broad range of irradiation are responsible for the effectiveness of pulsed light treatments on bacteria. Short wavelengths of light are responsible for damaging DNA, while longer wavelength high-intensity radiation would induce thermal harm to microorganisms (Food and Drug Administration, 2013). While limited studies exist, several investigators have shown that pulsed light treatments may be potential alternatives to reduce the microbial load in fruit juices (Pataro et al. 2011; Palgan et al. 2011; Ferrario et al. 2013).

Sonication

Sonication is the process by which microscopic air bubbles are forced to move, increase in volume, and collapse quickly, thereby causing shock waves which agitate particles in fluids. Hughes and Nyborg (1962) have suggested, the mechanism of disruption of the bacterial cells in a laboratory setting is related to intracellular cavitation. While sonication by itself can't guarantee the minimum requirements for microbial safety, it does have the potential to be combined with other preservation treatments to serve as a hurdle technology (Feng and Yan, 2011).

High Pressure Processing

High pressure processing is a novel non-thermal processing method that has demonstrated great potential in reducing microbial populations in foods while still maintaining natural attributes of the food. Hite (1899) was the first to report the effect of high pressure on milk preservation. Coagulation of egg albumen via high pressure processing was reported by Bridgman (1914) and the efficacy of pressure to eliminate microorganisms in fruits and vegetables was reported by Hite (1914). In 1987, Hayashi et al. introduced High Pressure Processing treatment to the food industry by examining proteolysis of beta-lactoglobulin in milk whey when subjected to high pressure. In 1990, fruit jams were the first high pressure processed foods to be commercialized. As high pressure has increasingly captured the attention of the food industry, more and more products such as jams, hams, sausages, instant rice packs, shucked oysters, clams, and smoothies (fruit beverages) are now being high pressure processed (San Martin et al. 2002).

High pressure processing is a non-thermal processing technique designed to produce a high quality product with limited processing, while extending shelf-life (Hoover, 1993; Knorr, 1993). This technology has several advantages when compared to thermal processing such as, pressure is

evenly (isostatic) and instantly transmitted throughout the product (Kelly, 2000; Smelt, 1998). It inactivates pathogenic and spoilage organisms while imparting minimal color change, limited flavor change, and mild texture change in foods (Knorr, 1993; Cheftel, 1995). High pressure processing is also able to preserve freshness because covalent bonds are not broken (Farkas and Hoover, 2000) and many nutrients including heat labile vitamins are preserved. Pressure can be applied at room temperature and thus eliminate thermally induced off-flavors. High pressure processing can be used to process liquid food products in semi-continuous equipment and liquid and solid foods in batch equipment. Pressures between 300 and 600 megapascals (MPa; 43,500 to 87,000 psi) have shown the capability of inactivating microorganisms while still maintaining fresh-like qualities of food products (Hoover et al., 1989; Stewart and Cole, 2001). As previously mentioned, high pressure processing is isostatic, meaning the pressure being exerted is instantly and uniformly being transmitted in addition to being adiabatic (little variation in temperature with increasing pressure regardless of food size and shape) (Smelt, 1998; Wilson et al., 2008). The even distribution of pressure prevents the deformation or heating of foods, thereby preserving its organoleptic properties.

High Pressure Processing is advantageous to the food industry because low-energy covalent bonds are unaltered and do not break when exposed to typical pressures for food. As a result, molecules (primary structures) such as fatty acids or proteins remain unbroken (Considine et al., 2008). Secondary and tertiary structures of proteins that are maintained by ionic bonds, hydrophobic interactions and Van der Waals forces are altered and disrupted allowing for a reduction in volume (Aymerich et al., 2008; Considine et al., 2008; Heremans, 1995; Ross et al., 2003). Changes, such as gelling, are observed in large macromolecules, like starch. Low molecular weight compounds such as vitamins, amino acids and flavor molecules are not substantially

affected by high pressure and thus organoleptic and nutritional properties are minimally modified (Aymerich et al., 2008; Farkas and Hoover, 2000). High Pressure Processing applications for the food industry are focused toward functional capacity and structure changes of proteins (Huppertz et al., 2006; San Martin et al., 2002), enzyme activity changes (Lopez-Malo et al., 1998), heat transfer (Otero and Sanz, 2003), combination of dehydration process (Ade-Omowaye et al., 2001), and compound extraction systems (Jun, 2006).

While the initial startup cost of high pressure processing may be high, the operating cost of product pressurized at 600 MPa has been estimated to be roughly $0.19 (US dollars) per kilogram (Aymerich et al., 2008). High pressure units that are available for commercial use are currently manufactured and sold by several companies but the main ones are: Hiperbaric (Spain), Avure Technologies and Multivac (USA), Stansted Fluid Power (United Kingdom) (Avure Technologies, 2015; Multivac, 2015; Hiperbaric, 2015; Stansted Fluid Power, 2015; Lavieri, 2013).

While high pressure processing has many advantages when utilized to process food products, it does have a few pitfalls when compared to thermal processing. As previously mentioned, the initial capital that must be invested in fairly sizeable. A commercial scale, high pressure vessel can cost anywhere from $500,000 to $2.5 million dollars depending upon capacity of the equipment and the extent of automation (Ramaswamy et al., 2004). High capital costs can be enough to prevent a company from utilizing this technology. Additionally, high pressure processing equipment is designed for only batch style processing as opposed to continuous line processing. This design limits the amount of volume that can be produced. As food industries are trying to produce as much safe product as possible, this technology may not be suited for all companies because a batch a style process requires loading a product into the vessel, pressurizing

the product, and then unloading the product. Depending on the product and pressure, some product may take up to 5 minutes to process (Murasaki-Aliberti et al., 2009). Shelf life of products is vital when determining what processing method to use. Certain high pressure processed products may not have as long of a shelf life when compared to thermally processed products (Ferrari et al., 2011). This is partly attributed to high pressure processing not having the ability to inactivate all enzymes, such as phenol oxidase, that cause browning or other effects that cause degradation (Keenan et al., 2012; Martinez and Whitaker, 1995).

Factors influencing bacterial resistance to high pressure

Experiments performed in model systems demonstrate that the physiological state of bacterial populations, undergoing pressurization, influence resistance to HPP (Rendueles et al., 2011). During the logarithmic phase, bacteria are more susceptible to inactivation as compared to the stationary phase (Hayman et al., 2007**,** Linton et al., 2001; Manas and Mackey, 2004; Pagan and Mackey, 2000). Incubation temperatures of bacterial populations also play a role of resistance to HPP (Bull et al., 2005). Another factor that aids in protecting microbial populations against HPP is the synthesis of proteins. In general, proteins provide protection against harsh conditions like high salt concentrations, acid environments, oxidative stress, and extreme temperatures (Considine et al., 2008; Wemekamp-Kamphuis et al., 2002; Wemekamp-Kamphuis et al., 2004). It has been reported that in *Listeria monocytogenes*, composition of the cell membrane, stationary phase and stress proteins affect resistance to pressure (Hayman et al., 2007). Microbial populations in foods are quite diverse and are in different physiological states and the ability for high pressure processing to inactivate microorganisms is influenced by the environmental conditions in which bacteria reside. Therefore, results obtained from model systems should be validated in “real food” products. The chemical composition of food play an important role in aiding microbial resistance

to HPP because fats, proteins, minerals, and sugars all provide microorganisms with a layer of protection (Black et al., 2007; Hauben et al., 1998; Molina-Hoppner et al., 2004). Foods that are high in nutrients such as milk or poultry meat can protect microorganisms (Patterson et al., 1995). Interestingly, in regards to the protective effect of fat against high pressure (baroprotective), contradictory results have been described in the literature. Some authors observed vegetative bacteria were protected against high pressure in an environment with increasing fat content (Styles et al., 1991; Garcia-Graells et al., 1999), while other authors did not see this difference (Garcia-Risco et al., 1998; Gervilla et al., 2000). In a study conducted by Gervilla et al. (2000), Ringer solution and milk with varying fat content (0, 6, 50% fat) was inoculated with *E. coli*, *Pseudomonas fluorescens*, *Listeria innocua*, *Staph aureus*, or *Lactobacillus helveticus* and were pressurized between 100 to 500 MPa at 4, 25, and 50 °C. It was reported that milk samples with varying fat content (0, 6, 50% fat) indeed had a baroprotective effect compared to the Ringer solution; however, samples with 6 and 50% fat content did not display an advanced protective effect in all pressurized treatments or for all microorganisms.

Based on multiple studies it has been shown that the effect of high pressure on microorganism is influenced by temperature (Ludwig et al., 1992, Sonoike et al., 1992, Moerman et al., 2001). Bacterial resistance decreases when food products are processed at chilled or moderately heated temperatures (Buckow and Heinz, 2008). The synergistic effect between low or subzero temperatures and high pressure processing on the inactivation of vegetative bacterial cells has been reported by several authors (Sonoike et al. 1992, Takahashi 1992, Hashizume et al. 1995, Hayakawa et al. 1998, Perrier-Cornet et al. 2005). Perrier-Cornet et al. (2005) demonstrated a slight decrease in a microbial population when high pressure was combined with ambient temperature, whereas, under the same conditions at low or subzero temperatures, the microbial

population was completely inactivated. It is important to note that this reported synergistic effect is highly dependent on the type of microorganism (Takahashi, 1992). The ability for HPP to have more of an effect at lower temperatures is likely attributed to membrane structure changes and fluidity through hydrophobic interactions and weakening and crystallization of phospholipids (Cheftel, 1995).

The effect of pH of the media used in combination with high pressure processing against microorganisms has been heavily studied. In one group of studies, it was observed that media with pH ranging from 3 to 8 did not influence high pressure inactivation of the following fungi: *Saccharomyces bayanus*, *S. cerevisiae*, *Mucor plumbeus*, *Zygosaccharomyces bailii*, and *Rhodotorula rubra* (Ogawa et al. 1990, Horie et al. 1991, Oxen and Knorr 1993, Pandya et al. 1995, Reyns et al. 2000). In a separate set of studies using bacterial pathogens (*Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli* O157:H7, and *Salmonella* spp.), it was observed that pH of media ranging from 4 to 6 substantially impacted the efficacy of HPP (Mackey et al. 1995, Stewart et al. 1997, Alpas et al. 2000). Based on the results from the two different groups of experiments, it is suggested that the combined antimicrobial effect of pH and high pressure processing is dependent on the type of microorganism subjected to high pressure processing.

Water activity plays an important role in the determination of cellular damage during pressurization. It has been observed that lower water activity of the food matrix, increases microbial resistance to high pressure processing (Black et al., 2007a; Black, et al., 2007b; Hayman et al., 2008; Patterson, 2005). While it is not entirely understood why lower activities protect microorganisms against HPP, Hayman et al. (2008) have proposed, in the case of *Listeria monocytogenes*, that low water activity results in stabilization of proteins, which prevents

denaturation of proteins and prevents cell death during high pressure processing. Moussa et al. (2009) evaluated the influence of cell hydration on the ability of *Saccharomyces cerevisiae* CBS 1171 to survive high pressure processing. The water activity of inoculated samples ranged between 0.11 and 0.99 and pressure treatments (600 MPa) were applied for 10 min, 24 h, and 6 days. It was observed that samples with water activities of 0.71 and below were completely protected against all pressure treatments. Aerobically dehydrated cells survived for 6 days at 600 MPa, despite the water activity levels being high (up to 0.94). When water is available in large quantities, high pressure causes membrane permeabilization, leading to uncontrolled leakage and ultimately cell death during prolonged exposure times under pressure (Moussa et al., 2009).

Effect of HPP on microorganisms

HPP has been quite useful as a food preservation technology because it allows microbial populations to be destroyed, thereby substantially increasing shelf-life and improving food safety (Considine et al., 2008). In general, high pressure processing, at ambient temperature, inactivates certain enzymes and destroys vegetative cells (Simpson and Gilmour, 1997). In addition, organoleptic properties are minimally changed (Farkas and Hoover, 2000; San Martin et al., 2002). While high pressure processing has been shown to inactivate microorganisms, factors such as: level of pressure, pressurization time, temperature, pH, water activity, cell growth phase, type of microorganisms, and suspending solution influence the ability of high pressure to inactivate microorganisms (Patterson et al., 1995 and McClements et al., 2001). Various studies have been conducted and have shown that several factors such as sodium chloride and sugars in the media and pH influence the effect of high pressure processing on the inactivation of microorganisms (Horie et al. 1991, Palou et al. 1997, Van Opstal et al. 2003, Molina-Hoppner et al. 2004).

Microorganisms that are structurally less complex, like prokaryotes, tend to exhibit a stronger resistance to HPP's destructive effect when compared to eukaryotes (Yuste et al., 2001). Parasites and protozoa can be eliminated at relatively low pressures. At pressures ranging from 100 to 400 megapascals, *Toxoplasma gondii* (Lindsay et al., 2006), *Anisakis simplex* (Brutti et al., 2010; Molina-Garcia and Sanz, 2002) *Cryptosporidium parvum* (Collins et al., 2005), *Trichinella spiralis* (Noeckler et al., 2001), and *Ascaris* (Rosypal et al., 2007) can be eliminated. Yeasts and molds have demonstrated average resistance to high pressure (Palou et al., 1998b; Palou, et al., 1997; Shimoda et al., 2002). Most bacterial spores are resistant to high pressure. *Clostridium botulinum* and *Bacillus* species spores have been shown to be resistant to pressures greater than 1000 MPa at room temperature (Margosch et al., 2006, Margosch et al., 2004). Unlike bacterial spores, mold mycelia are quite susceptible to high pressure (Chapman et al., 2007; Smelt, 1998). Palou et al. (1998a) demonstrated at 689 MPa, ascospores of *Byssochlamys* spp. are able to show resistance to high pressure and an increase in processing temperature is required in order to obtain a significant loss in viability.

Vegetative foodborne pathogens can demonstrate a log reduction of up to 8 with pressures as low as 300 MPa when combined with a temperature of 50 °C for 5 minutes (Alpas et al., 1999). While there are significant differences of log reduction units between pathogens, it has also been observed that there are differences between strains of pathogens belonging to the same genus or species (Alpas et al., 1999; Benito et al., 1999; Bull et al., 2009; Garcia-Graells et al., 2000). Spores have demonstrated great resistance against high pressure processing (Rendueles et al., 2011). Species such as *Bacillus amyloliquefaciens* are capable of forming extremely high-pressure resistant spores and it has been suggested that it should be accepted as the target organism when developing high pressure processing standards (Margosch et al., 2004; Rajan et. al, 2006). In a

study conducted by Margosch et al. (2004), mashed carrots, inoculated with seven strains of *Clostridium botulinum,* were high pressure processed at pressures ranging from 600 to 800 MPa at temperatures ranging from 80 to 116 degrees °C. It was observed that spores of *Clostridium botulinum* were able to withstand 800 MPa for 16 minutes at 70 degrees celsius without a reduction in spore count. Similarly, with *Bacillus cereus*, a combination of pressures exceeding 600 MPa and temperatures exceeding 45 degrees celsius are required to demonstrate a loss in viability (Oh & Moon, 2003; Van Opstal et al., 2004).

Cell membrane damage is believed to be one of the most important factors responsible for bacterial death after bacterial cells are high pressure processed (Ritz et al., 2001, Russell, 2002). The cell membrane is believed to be the target of High Pressure Processing because HPP (Pagan and Mackey, 2000; Ritz et al., 2000; Ritz et al., 2002, Russell, 2002; Smelt, 1998) causes modification of membrane permeability as well as functionality disruption (Pagan and Mackey, 2000). Cell leakage has also been observed as a result of HPP. Shimada et al. (1993) observed, following high pressure processing, intracellular fluids of *Saccharomyces cerevisiae* were detected via absorbance in fluid suspending the cells. Gram-positive and gram-negative bacteria display varying degrees of resistance to HPP due to their difference in structural properties and chemical composition (Russell, 2002). It has been observed that Gram-positive bacteria are typically more resistant to HPP than Gram-negative bacteria (Shigehisa et al., 1991); however, it has been observed that there is some overlap. For example, *Leuconostoc dextranicum* displays a greater sensitivity to HPP than various gram-negative bacteria such as *Shigella flexneri*, *E. coli* and *Salmonella typhimurium*. In addition, the pressure resistant mutant of *E. coli* (LMM1010) is more resistant than various gram positive bacteria such as (*Listeria innocua*, *Lactobacillus plantarum*, and *Leuconostoc dextranicum*) (Syed et al., 2015; Wuytack et al., 2002). In addition to causing

membrane disruption, HPP also alters or inhibits ribosomes (Kaletunc et al., 2004), protein synthesis (Considine et al., 2008; Simpson & Gilmour, 1997; Wouters et al, 1998) and enzyme activity, and DNA-enzyme complexes structure (DNA degradation) (Chilton et al., 1997).

However, it has been shown that some bacterial cells are able to survive even after being high pressure treated at lethal levels (Patterson et al. 1995, Simpson and Gilmour 1997, McClements et al. 2001, Ritz et al. 2001, Chen and Hoover 2003). Bacterial cells are capable of repairing the site of injury during storage and may potentially proliferate after sub-lethal injury induced by high pressure processing (Jordan et al., 2001; Russell, 2002). During storage at various temperatures in broth, milk, and ground pork within 6 h to 4 weeks, it has been reported that pathogenic and spoilage bacteria that were sub-lethally injured because of high pressure processing were able to be recovered (Ellenberg and Hoover, 1999; Chilton et al., 2001; Bozoglu et al., 2004; Bull et al., 2005). Bozoglu et al. (2004) demonstrated that despite injured cells being undetectable immediately following high pressure processing, they were able to be detected after repairing themselves within 1 to 15 days. The recovery of *E. coli* in nutrient-rich medium (Tryptic Soy Broth) following incubation after high pressure treatment was evaluated by Koseki and Yamamoto (2006). It was observed that *E. coli* was able to significantly recover when stored at 25 °C. Healthy, viable microbial cells typically grow on and are countable on nonselective and selective media, whereas cells that have been subjected to stress or sub-lethal injury are able to grow on nonselective media but do not grow on selective media (Comas and Rius, 2009). Stressed cells display changes in morphology and structure such as membrane separation from the cell wall, cell lengthening, compression of gas vacuoles (Patterson, 2005) and nuclear material condensation (Manas and Mackey, 2004; Wouters et al., 1998). It is important to account for injured or stressed microorganisms because under favorable conditions they can resuscitate and resume normal

functions, thereby putting public health at risk. During the repair period, growth capabilities are restored, cellular modifications are reversed, and cell constituents that were lost are restored (Syed et al., 2015). To ensure safety of high pressure processed foods, it is important to accurately estimate bacterial recovery and it is critical to determine the possible presence of sub-lethally injured microorganisms in HPP foods as these organisms may potentially resuscitate if given favorable conditions for repair.

High pressure processing has been evaluated as an alternative processing method to thermal processing to inactivate microorganisms and produce high quality and safe food. High pressure processing is a promising technology to produce healthy, natural, clean label foods. The effectiveness of high pressure is primarily dependent on the pressure applied and on the holding time. Microbial resistance will vary based on the organism type as well as the food matrix. While HPP has demonstrated its ability to inactivate microorganisms, future experiments need to be conducted to evaluate the effects of HPP under temperatures below subzero on inactivation of microbes and retention of organoleptic properties. In addition, it will be beneficial to the food industry to continue to explore other potential food applications for high pressure processing.

Essential oils derived from plant sources

Essential oils (EOs), also referred to as volatile or ethereal oils (Guenther, 1948), are oily substances that contain various aromas and are isolated from plants or plant material. Methods such as fermentation, expressions, extraction or enfleurage can be used to obtain essential oils but the most common method of isolation is steam distillation (Van de Braak and Leijten, 1999). The term “essential oil” stems from Paracelsus von Hohenheim, as he was credited with naming the effective component of a drug named *Quinta essential* in the 16th century (Guenther, 1948). Approximately 3000 EOs are known and roughly 300 (mostly used as flavors and fragrances) are

commercially important (Van de Braak and Leijten, 1999). Essential oils are considered to be secondary metabolites of plants and possess a broad spectrum of antimicrobial properties (Guenther, 1948; Boyle, 1955; Fraenkel, 1959; Tajkarimi et al., 2010; Deans and Ritchie, 1987; Oussalah et al., 2007). Other studies have demonstrated that essential oils possess antiparasitic (George et al., 2009), antiviral (Schnitzler et al., 2011), insecticidal (Essam, 2001; Kim et al., 2003), antimycotic (Azzouz and Bullerman, 1982; Akgul and Kivanc, 1988; Jayashree and Subramanyam, 1999; Mari et al., 2003) and antioxidant (Brenes and Roura, 2010) properties.

Currently, essential oils are heavily used as flavorings in food, as perfumes and as pharmaceuticals (Bauer and Garbe, 1985; Van Welie, 1997; Van de Braak and Leijten, 1999). Essential oils are not only used as antimicrobials in foods but are also used as dental root canal sealers (Manabe et al., 1987), antiseptics (Bauer and Garbe, 1985; Cox et al., 2000) and feed supplements for weaned piglets and lactating sow (Van Krimpen and Binnendijk, 2001; Ilsley et al., 2002). As previously mentioned, essential oils are primarily used in the food industry as flavorings; however, since essential oils possess preservative characteristics it is worth investigating their full potential to be used in foods. Extensive knowledge (such as target organisms, mode of action, minimum inhibitory concentration, effect of the food matrix on the antimicrobial) will be required to fully understand the application of essential oils (Hyldgaard et al., 2012).

Essential oil production

Currently the most common method for producing essential oils on a large commercial scale is steam distillation. Moyler (1998) demonstrated essential oil extraction by way of using high pressure and low temperature liquid carbon dioxide. While this method produced a natural organoleptic profile, it is also more expensive. The difference in organoleptic profile is a result of

a difference in the make-up of oils acquired through solvent extraction compared to distillation (Burt, 2004). The difference in the composition of oils may also influence the antimicrobial properties.

Classes of essential oil active compounds

On a daily basis, plants are producing various compounds that have a broad spectrum of antimicrobial activity. Some of these compounds are naturally present and available and some are produced as a result of foreign invasion (i.e microorganisms) or physical injury (Roller, 2003). Since essential oils are complex mixtures of up to 45 different constituents, it is ultimately useless to try and identify the most active antimicrobial compound (Delaquis et al., 2002; Djenane et al., 2011; Espina et al., 2011) especially since the composition of a specific essential oil can change based on the extraction method and season of harvest (Nannapaneni et al., 2009; Pereira and Meireles, 2010; Sanchez et al., 2010; Demuner et al., 2011; Djenane et al., 2011; Paibon et al., 2011). Essential oils are a diverse family of organic compounds and the active compounds can be divided into four groups or classes based on their chemical structure. These four classes include: terpenes, terpenoids, phenylpropenes, and "others."

Terpenes are hydrocarbons that result from the combination of several isoprene units (C_5H_8). Terpenes are produced in the cytoplasm of plant cells via the mevalonic acid pathway beginning with acetyl-CoA (Hyldgaard et al., 2012). Terpenes possess a hydrocarbon backbone that can be arranged into cyclic structures by an enzyme known as cyclase and can form monocyclic and bicyclic structures (Caballero et al., 2003). Terpenes in general do not have the highest degree of antimicrobial activity. Bagamboula et al. (2004) demonstrated p-cymene, major compound in thyme, had no effect against *Shigella sonnei* and *Shigella flexneri* at a concentration of 85,700 μg/mL. In an experiment conducted by Dorman and Deans (2000), it was observed that

limonene, α-pinene, β-pinene, δ-3-carene, (+)-sabinene, and α-terpinene showed little to no antimicrobial activity against 25 genera of bacteria associated with diseases and death in plants and animals, food poisoning and spoilage. Against *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus*, α-pinene, β-pinene, *p*-cymene, β-myrcene, β-caryophyllene, limonene, and γ-terpinene demonstrated a similar trend of little to no antimicrobial effect (Koutsoudaki et al., 2005). Rao et al. (2010) investigated the effect of cymene and γ-terpinene on *Saccharomyces cerevisiae* and it was observed that cymene and *γ*-terpinene were ineffective at inhibiting *Saccharomyces cerevisiae.*

The second major class of active compounds of essential oils is terpenoids. Terpenoids are terpenes that are biochemically modified through enzymes adding oxygen molecules and moving or removing methyl groups (Caballero et al., 2003). Terpenoids can be broken down into seven groups that include: alcohols, esters, aldehydes, ketones, ethers, phenols, and epoxides. Some common examples of terpenoids include: thymol, carvacrol, linalool, linalyl acetate, citronellal, piperitone, menthol, and geraniol *(*Hyldgaard et al., 2012). It has been shown that the antimicrobial activity of most terpenoids is attributed to the hydroxyl group of phenolic terpenoids and the presence of delocalized electrons. For example, the antimicrobial activity, hydrophobicity, and bacterial membrane interaction of carvacrol is affected when the hydroxyl group of carvacrol is exchanged with methyl ether (Veldhuizen et al., 2006). Derivatives of carvacrol, carvacrol methyl ether and *p*-cymene, have a lower antimicrobial activity than carvacrol (Dorman and Deans, 2000; Ultee et al., 2002; Ben Arfa et al., 2006). Terpenoids possess great potential to be used in the food industry because they are able to inhibit a broad spectrum of microorganisms. The most well known and most active compounds of the terpenoids class are thymol and carvacrol. Phenylpropenes are a subgroup of the organic compounds known as phenylpropanoids.

Phenylalanine in plants is the precursor for which phenylpropanoids are synthesized. Phenylpropenes represent only a small fraction of essential oils, with the most recognized ones being cinnamaldehyde, vanillin, safrole, eugenol, and isoeugenol. In a study conducted by Laekeman et al. (1990), it was observed that free hydroxyl groups are important for antibacterial activity (not including yeast) of eugenol and isoeugenol when compared to chemically similar molecules. Other factors that influence the antimicrobial effectiveness of phenylpropenes include: bacterial strains, type and quantity of substituents on the aromatic ring, growth medium, temperature, pH, etc. (Pauli and Kubeczka, 2010).

The last class of active essential oil compounds are classified as "other." Degraded products originating from unsaturated fatty acids, glycosides, terpenes, lactones, and sulfur and nitrogen containing compounds can be found in essential oils (Caballero et al., 2003). Allicin and allyl isothiocyanate (AITC) are two examples of sulfur and nitrogen containing compounds. Allicin is a compound found in garlic and functions as a defense mechanism in plants (Ankri and Mirelman, 1999). Allicin has a pronounced aroma of garlic and displays antifungal, antiparasitic, antiviral, and antibacterial properties (Kyung, 20111). Allicin has been shown to be equally effective against Gram-negative and Gram-positive bacteria and is bactericidal with LD_{50} values ranging between 3 and greater than 100 μg/mL (Cavallito and Baily, 1944; Ankri and Mirelman, 1999). Allyl isothiocyanate is commonly found in mustard oils, does not contain phenolic components and can constitute approximately 90% of the oil composition (Ward et al., 1998). Allyl isothiocyanate has been shown to possess a high bactericidal effect against food spoilage microorganisms and food pathogens such as *E. coli* O157:H7, *S. typhimurium*, *L. monocytogenes*, and some aerobic Gram-negative spoilage bacteria (Luciano and Holley, 2009; Delaquis, 1997).

Allyl isothiocyanate is also effective at inhibiting a wide variety of fungi (Delaquis, 1997; Nielsen and Rios, 2000).

Mechanism of action of essential oils

While the antimicrobial efficacy of essential oils have been evaluated, the mechanism of action of essential oils has not been greatly studied (Lambert et al., 2001). Due to the large group of chemical compounds present within essential oils, it is unlikely that the antibacterial activity is attributed to one specific mechanism (Skandamis et al., 2001; Carson et al., 2002). It is important to note that essential oils are hydrophobic, which allows for partitioning in the lipids of bacterial cells and mitochondria resulting in structure disturbance and rendering the cell more permeable (Knobloch et al., 1986; Sikkema et al., 1994). With a cell becoming more permeable, leaking of ions and other cell contents can occur (Oosterhaven et al., 1995; Gustafson et al., 1998; Helander et al., 1998; Cox et al., 2000; Lambert et al., 2001; Skandamis et al., 2001; Carson et al., 2002; Ultee et al., 2002). Bacterial cells can tolerate leakage without loss of viability up to a certain point; however, with excessive cell content loss or loss of critical molecules death of the cell will occur (Denyer and Hugo, 1991). For essential oils possessing a high quantity of phenolic compounds, the mechanism of action would be similar to other phenolics and would be attributed to the cytoplasmic membrane disruption, disruption of proton motive force (PMF), electron flow, active transport, and coagulation of cell contents (Denyer and Hugo, 1991a; Sikkema et al., 1995; Davidson, 1997). The antibacterial activity and mode of action of essential oil components is dependent on the chemical structure of the essential oil components. For example, several studies have confirmed the importance of the hydroxyl group in phenolic compounds like carvacrol and thymol (Knobloch et al., 1986; Dorman and Deans, 2000; Ultee et al., 2002). The relative position of the hydroxyl group on the ring does not appear to play a pivotal role in influencing antibacterial

activity as evidenced by the antibacterial effect of thymol against *B. cereus*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* being comparable to carvacrol (Lambert et al., 2001; Ultee et al., 2002). For essential oils that do not contain phenolics, it has been observed that the type of alkyl group influences the activity (alkenyl>alkyl) (Burt, 2004).

Not only do components of essential oils act on the membrane, but EO components have also been shown to act on embedded cell proteins in the cytoplasmic membrane (Knobloch et al., 1989). The cytoplasmic membrane contains enzymes, such as ATPases, that are surrounded by lipid molecules. Cyclic hydrocarbons could potentially act on the lipid molecules. It has been hypothesized that lipophilic hydrocarbon molecules could accumulate in the lipid bilayer, thereby disrupting the lipid-protein interaction. Another proposed mechanism is the lipophilic compounds directly interact with the hydrophobic parts of the protein (Juven et al., 1994; Sikkema et al., 1995). Amino acid decarboxylases in *Enterobacter aerogenes* have been shown to be inhibited by cinnamon and components of cinnamon and it has been proposed that the mechanism of action is protein binding (Wendakoon and Sakaguchi, 1995).

Food preservation by essential oils

A wide variety of essential oil components have been accepted by the European Commission as well as the Food and Drug Administration (FDA) for their use as food product flavorings. Some examples include: thymol, linalool, eugenol, carvone, vanillin, cinnamaldehyde, carvacrol, citral and limonene. These compounds are considered safe and present no risk to consumer health. With the growing trend of consumers wanting all natural products, it is important for more natural ingredients to be incorporated into product formulations. Despite the relative success of essential oils and their constituents inhibiting microorganisms *in vitro*, their use as a food preservative has been limited due to having to use a large concentration in foods to inhibit

microorganisms. In numerous food products, hydrophobic essential oil compounds are disrupted by interactions with components of food such as starch (Gutierrez et al., 2008), and proteins (Cerrutti and Alzamora, 1996; Kyung, 2011). In addition, other factors such as pH (Juven et al., 1994), temperature (Rattanachaikunsopon and Phumkhachorn, 2010), and presence of microorganisms (Somolinos et al., 2010) play an important role in the efficacy of essential oil compounds. Therefore, it is imperative that essential oils and their constituents be first tested *in vitro* to serve as a baseline before being added to a food product.

A challenge faced when adding essential oils and constituents to food products is the aroma. The aroma can cause negative organoleptic effects that can be deemed unacceptable by consumers (Lv et al., 2011). This can be difficult when concentrations of essential oils must be increased to compensate for their interactions with food constituents. In this regard, essential oils have limited application. Various approaches can be used to address this issue. One strategy is to use essential oils in active packaging as opposed to a product ingredient (Hyldgaard et al., 2012). Essential oils can be stored in polymers of edible and biodegradable coatings or sachets via encapsulation and can be released slowly to the food surface or headspace of packages (Pelissari et al., 2009; Sanchez-Gonzalez et al., 2011). The main advantage of encapsulating essential oils in films or edible coatings is that the rate of diffusion of the agents away from the food product can be minimized , allowing for active compounds to be maintained in the headspace or on the product surface for prolonged periods of time (Phillips and Laird, 2011; Sánchez-González et al., 2011). Another strategy for reducing negative organoleptic effects is to lower the concentration of essential oils by applying them in combination with other antimicrobial compounds (Nguefack et al., 2012) or with another processing intervention that provides a synergistic effect. Since it is known that synergies occur for combinations of essential oils, it opens the door for future

antimicrobial blends that can be produced and incorporated into various food products without negative organoleptic effects.

Geraniol

Geraniol, 3,7-dimethyl-2,6 and 3,6-octadien-1-ol, is an acyclic monoterpenoid alcohol. Geraniol is characterized as having a rose-like odor and at 10 ppm, the taste is described as a sweet floral rose, citrus with fruity, waxy nuances (Burdock, 2010). Geraniol is a clear to pale yellow oil that is soluble in most organic solvents but not in water. Under the Code of Federal Regulations Title 21, part 182, Geraniol is a flavoring substance that is generally recognized as safe (FDA, 2014). Geraniol is commonly found in essential oils and occurs in sources such as citronella oil (24.8%) (Rajeswara Rao et al., 2004), palmarosa oil (53.5%) (Dubey and Luthra, 2001), rose oil (44.4%) Baydar and Baydar, 2005), ninde oil (66.0%) (Baser et al., 2005), and *Monarda fistulosa* (>95%) (Simon et al., 1986). In vitro and in vivo studies have shown that Geraniol is capable of demonstrating antitumor activity against hepatoma, melanoma, and murine leukemia cells (Burke et al., 1997; Yu et al., 1995a, b). In a survey of European market consumer products, it was revealed that Geraniol was in 76% of investigated deodorants, was included in 41% of domestic and household products and was included in 33% of cosmetic formulations related to natural ingredients and its production is greater than 1000 metric tons annually (Rastogi et al., 1996; Rastogi et al., 1998; Rastogi et al., 2001; Chen and Viljoen, 2010). In addition, Geraniol has been shown to be effective at repelling insects (Barnard and Xue, 2004) as well as having antimicrobial properties (Bard et al., 1988). Geraniol is a natural compound that has various applications and much research still needs to be conducted to discover its full antimicrobial capabilities.

Chemical structure and biosynthesis

The molecular formula of geraniol is $C_{10}H_{18}O$ and its molecular weight is 154.25 g/mol. "Geraniol" is composed of two cis-trans isomers known as nerol (cis) and geraniol (trans) (figure 1). Nerol has been isolated from neroli oil, while Geraniol has been isolated from Palmarosa oil (Bedoukian, 1986; Clark, 1998). Geraniol is generated from geranyl diphosphate through an ionization dependent reaction mechanism (Bohlmann et al., 1998). Geranyl diphosphate is produced through a condensation reaction between dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) (Croteau and Purkett, 1989). Generally, biosynthesis of geraniol is through the HMG-CoA reductase pathway (mevalonate pathway), but is has been shown that in some plants geraniol can be synthesized through the non-mevalonate pathway (Luan and Wust, 2002).

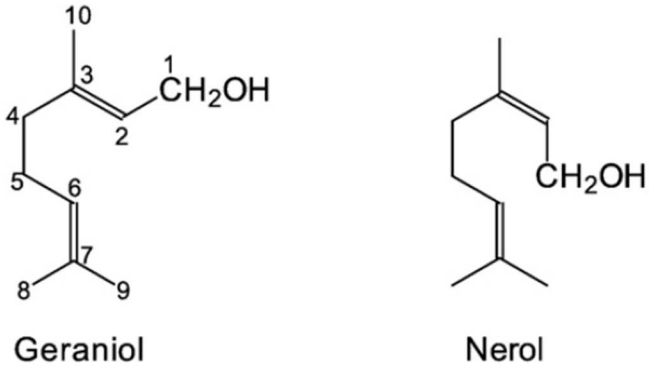

Figure 1. Chemical structure of Geraniol and Nerol. (Adapted from Chen and Viljoen, 2010).

Pest repellant properties

Due to their repellent, antifeedant, and insecticidal properties, research has demonstrated that essential oils are slowly emerging as mosquito control products (Barnard and Xue, 2004). In

addition, their low toxicity to mammals and biodegradability has hastened the push for their development. To test the acaricidal effects of geraniol, Jeon et al. (2009) compared geraniol to the commercial acaricide, benzyl benzoate, against the food mite, *Tyrophagus putrescentiae*. Against the food mite, geraniol was more effective than benzyl benzoate. A 50% lethal dose value of 1.95 $\mu g/cm^3$ was observed for geraniol, whereas 1.27 $\mu g/cm^3$ was observed for benzyl benzoate. In a separate study, Traina et al. (2005) evaluated the acaricidal activity of α-pinene, geraniol, limonene and *p*-cymene (all monoterpenes) against the ear mite *Otodectes cynotis*. It was observed that geraniol, in a 5% solution, was effective against the ear mites when in direct contact. Against ticks on cows, Geraniol (1%), reduced the mean population (ticks per animal) by 98.4%, 97.3%, and 91.3% at days 7, 14, and 21 respectively (Khallaayoune et al., 2009).

Mosquitoes are a noteworthy health risk because they are capable of carrying several severe diseases. Omolo et al. (2004) demonstrated that geraniol is effective at repelling mosquitoes. Geraniol based natural repellents are commercially available in multiple countries. Candles containing geraniol demonstrated a higher degree of effectiveness in protecting humans indoors from being nipped by mosquitoes and sand flies in comparison to candles containing citronella and linalool (Müller et al., 2008). A study was conducted by Müller et al. (2009) to determine the degree of protection exhibited by commercial geraniol, linalool, and citronella candles against mosquitoes and sand flies. Against mosquitoes the repellency rate of a 5% geraniol candle was 85.4%. Candles containing 5% citronella or 5% linalool demonstrated a repellency rate of 29.0% and 71.1%, respectively. A similar trend was observed in sand flies. A 5% geraniol candle exhibited a repellency rate of 79.7%, whereas candles containing citronella or linalool displayed a repellency rate of 24.7% and 55.2% respectively. Geraniol candles were approximately 5 times as effective as citronella candles and roughly twice as effective as linalool candles at repelling

mosquitoes and sand flies indoors. In a study conducted by Hao et al. (2008), the host-seeking behavior of the *Aedes albopictus* mosquito was evaluated in an area containing vapors of citronellal, citral, anisaldehyde, eugenol, and geraniol. The host-seeking ability of nearly 100% of the mosquitoes seemingly vanished after 48 hours of exposure geraniol at a concentration of 0.250 µg/ml geraniol.

Parasiticidal properties

Parasites that infect humans can cause zoonotic disease such as anisakiasis (Chen and Viljoen, 2010). It has been demonstrated *in vitro* and *in vivo*, that various monoterpenes display substantial anti-helminthic activity (Hierro et al., 2006; Navarro et al., 2008). In a study conducted by Leela et al. (1992), the essential oil of *P. graveolens* and its major constituents, geraniol, citronellol, and linalool, was evaluated against the root-knot nematode, *Meloidogyne incognita*. Geraniol was determined to display the greatest nematicidal activity. In a separate study, Kumaran et al. (2003), evaluated the anthelmintic activity of the Palmarosa oil (essential oil extracted from *Cymbopogon martini*) and geraniol (a main constituent) against the nematode, *Caenorhabditis elegans*. Geraniol was determined to have a median effective dose (ED50) of 66.7 µg/ml. The results of the study suggest that geraniol is responsible for the anthelmintic properties of palmarosa oil. In addition to possessing nematicidal activity, geraniol has also been shown to exhibit larvicidal activity against *Contracaecum* (roundworms) (Barros et al., 2009) and against *Anisakis simplex* (marine nematodes) (Hierro et al., 2004).

Antimicrobial properties

Essential oils are becoming increasingly popular due to the fact that they are extracted from natural sources and are capable of exhibiting antimicrobial activity against a wide host of bacteria

and fungi. The solubility of the phospholipid bilayer of cell membranes influences the antimicrobial activity of essential oils (Knobloch et al., 1989). Monoterpenoid alcohols such as linalool, nerol, citronellol and geraniol have demonstrated a higher degree of antibacterial activity in comparison to antifungal activity (Suppakul et al., 2003). In a study conducted by Friedman et al. (2002), 96 essential oils and 23 oil compounds were evaluated for bactericidal activity against *Campylobacter jejuni*, *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella enterica*. With a bactericidal value of BA_{50} 0.10, geraniol was most effective against *Campylobacter jejuni.* Against *E. coli* O157:H7 and *S. enterica*, geraniol had a bactericidal activity value of BA_{50} 0.15, whereas against *Listeria monocytogenes* the bactericidal activity value was BA_{50} 0.28. As a gas, geraniol is capable of displaying antibacterial effects against commonly known pathogens that affect the respiratory tract such as *Streptococcus pneumonia*, *Streptococcus pyogenes*, *Haemophilus influenza*, and *Staphylococcus aureus* (Inouye et al., 2001). Scortichini and Rossi (2008) assessed the antimicrobial efficacy of geraniol, in tube cultures, against seven strains of the causal agent of "fire blight" of rosaceous plants known as *Erwinia amylovora*. For 24 h, geraniol, ranging from 600-1500 mg/l, inhibited all *Erwinia amylovora* strains at a concentration of 1×10^5 cfu/ml.

Antifungal properties displayed in palmarosa oil (Cymbopogon martinii) are mainly attributed to its geraniol content (Prashar et al., 2003). Against *Saccharomyces cerevisiae*, antifungal action of palmarosa oil occurs in two stages. In the first stage, membrane disruption occurs via passive entry of the oil into the plasma membrane. In the second stage, the oil accumulates in the plasma membrane resulting in cell growth inhibition. The antifungal activity is attributed to combined membrane effects such as increased bilayer disorder and ion leakage. These effects disrupted the osmotic balance of the cells through the loss of ions resulting in inefficiencies

of membrane-associated proteins ultimately leading up to cell growth inhibition and death.

Essential oils have been shown to have inhibitory effects against planktonic cells, but they are also capable of exerting an inhibitory effect on biofilms. More specifically, carvacrol, geraniol, and thymol can be utilized as potential biofilm inhibiting agents (Chen and Viljoen, 2010). Not only are some plant extracts capable of providing inhibitory effects individually, but also some are capable of providing synergistic activity against bacteria and fungi. Against *Trichophyton schoenleinii* and *Trichophyton soudanense*, the essential oil extracted from *Psilocybe graveolens*, containing geraniol and citronellol, displayed a strong synergistic effect with ketoconazole with fractional inhibitory concentrations indices (FIC) ranging from 0.18-0.38 (Shin and Lim, 2004). Rosato et al. (2007) demonstrated that the combination of the essential oil of *Psilocybe graveolens* and the antibacterial agent, norfloxacin, exhibited a synergistic effect against two strains of *Staphylococcus aureus* and *Bacillus cereus*. The FIC ranged from 0.38-0.50. Through the use of the essential oil extracted from *Helichrysum italicum*, the multi-drug resistance of gram-negative bacteria, *E. coli, Pseudomonas aeruginosa, Enterobacter aerogenes*, and *Acinetobacter baumannii* to chloramphenicol can be significantly reduced. By targeting efflux mechanisms, the active compound in *Helichrysum italicum*, geraniol, was able to increase of the efficacy of β-lactams, quinolones, and chloramphenicol (Lorenzi et al., 2009).

Antioxidant properties

Oxidation of amino acids, DNA, unsaturated lipids, and proteins is a result of free radicals and other reactive oxygen species (ROS). Free radicals and ROS are responsible for producing molecular changes related to cancer, aging, arteriosclerosis, Alzheimer's disease, diabetes and asthma (Gardner, 1997; Edris, 2007). In a study conducted by Choi et al. (2000), 34 citrus essential oils and their components were examined for radical scavenging activities. Geraniol displayed

scavenging activities against the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. Against tertiary-butyl hydroper-oxide stressed rat alveolar macrophages, geraniol demonstrated antioxidant potential (Tiwari and Kakkar, 2009). Cell viability significantly increased via geraniol and also displayed a 120% increase in glutathione, 45% increase in superoxide dismutase activity and mitochondrial membrane potential was restored. In comparison to the stressed cells, geraniol inhibited nitric oxide release and decreased lipid peroxidation. In addition, geraniol inhibited reactive oxygen species as well as provided protection against it.

Anticancer properties

Geraniol has demonstrated anticancer properties in both in vitro and in vivo models of human cancer (Chen and Viljoen, 2010). When used to treat pancreatic and other cancers, Geraniol exhibited chemotherapeutic activity (Burke et al., 1997). When evaluated against pancreatic tumors, the mechanism of action of Geraniol was determined to be apoptosis and increased expression of the proapoptotic protein Bak in cultural tumor cells of the pancreas (Burke et al., 2002). In a separate study, Wiseman et al. (2007) revealed that Cdk2 activity was reduced in addition to decreased expression of downstream cell cycle related proteins in human pancreatic adenocarcinoma cells because geraniol caused stoppage in the G_0/G_1 phase of the cell cycle. It was observed that mammary tumor multiplicity was inhibited in female Sprague-Dawley rats treated with geraniol for 2 weeks before starting with 7,12-dimethylbenz[α]anthracene and 22 weeks afterwards (Yu et al., 1995). It has also been observed that against hepatoma and melanoma cell growth, geraniol has antiproliferative properties (Polo and De Bravo, 2006).

Anti-inflammatory properties

The immunosuppressive properties of geraniol was evaluated using *in vitro* lymphocyte proliferation assays and *in vivo* in a rat cardiac allograft model (Ji. et al., 2002). It was revealed that geraniol possesses modest *in vitro* and *in vivo* immunosuppressive activity. The anti-inflammatory activity of select essential oils on neutrophil activation was assessed *in vitro* via determining the tumor necrosis factor-alpha (TNF-α)-induced adherence reaction of human peripheral neutrophils. It was discovered that at a concentration of 0.0125%, spearmint, geranium, lemongrass and their major constituents (ctiral, geraniol, citronellol, and carvone) suppressed TNF-α-induced neutrophil adherence (Abe et al., 2003).

Cinnamaldehyde

Cinnamaldehyde, (2E)-3-phenlprop-2-enal, is a phenylpropene aldehyde that was isolated in 1834 by Dumas and Peligot from the essential oil of cinnamon. Cinnamaldehyde is characterized as an organic compound that is a pale yellow, viscous liquid that occurs naturally in the bark of cinnamon trees and species of the genus *Cinnamomum*. It is responsible for giving cinnamon its flavor and odor. Cinnamaldehyde makes up 55-75%, 1-8%, and 70-95% of essential oil extracted from cinnamon bark, cinnamon leaf, and cassia, respectively (Ross, 1976). Under the Code of Federal Regulations Title 21, part 182, Cinnamaldehyde is a flavoring substance that is generally recognized as safe. In addition to being used as a flavoring substance, cinnamaldehyde is used in fragrances found in decorative cosmetics, fine fragrances, shampoos, toilet soaps and other toiletries as well as household cleaners and detergents (Cocchiara et al., 2005). Cinnamaldehyde is a natural compound that has multiple applications and ample research is still needed in order to determine the full scope of its antimicrobial properties.

Chemical Structure

The molecular formula of cinnamaldehyde is C_9H_8O and has a molecular weight of 132.16 g/mol. When extracted from cinnamon, cinnamaldehyde is more commonly referred to as trans-cinnamaldehyde (figure 2). Trans-cinnamaldehyde is comprised of a phenyl group that is attached to an unsaturated aldehyde. Trans-cinnamaldehyde constitutes approximately 65% of cinnamon (Lens-Lisbonne et al., 1987).

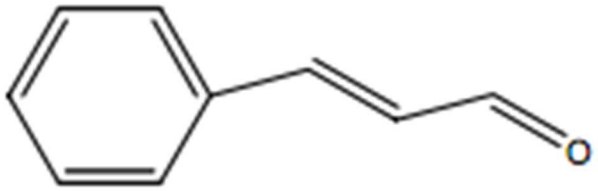

Figure 2. Chemical structure of Cinnamaldehyde. (Adapted from Cocchiara et al., 2005)

Antimicrobial properties and proposed mechanism of action

Aldehyde groups are reactive and contain the necessary qualities to covalently cross-link with DNA proteins through amine groups, which in turn interfere with their normal function (Feron et al., 1991). Cinnamaldehyde has displayed strong antimicrobial activity against microorganisms (Zaika, 1988). In 1887, Chamberland revealed cinnamon oil to be lethal against anthrax spores (Davidson et al., 1983). In a separate study, it was determined that cinnamon exhibited a wide inhibitory spectrum against bacteria and fungi in comparison to other extracts of spices and herbs at various pHs (Ueda et al., 1982). While the mode of action of cinnamaldehyde is inconclusive, it is believed there are three reactions that potentially occur: at small concentrations, cinnamaldehyde inhibits various enzymes involved in cytokinesis or less important cell function. At larger, but sub-lethal concentrations, cinnamaldehyde acts as an ATPase inhibitor, and at lethal

concentrations, cinnamaldehyde disrupts the cell membrane (Hyldgaard et al., 2012). Against *Bacillus cereus*, cinnamaldehyde was suggested to inhibit cytokinesis as a mode of action due to cells not separating although septa were present after division (Kwon et al., 2003). It has been shown that cinnamaldehyde binds to the FtsZ protein, which in turn inhibits its GTP dependent polymerization and thus prevents cell division (Domadia et al., 2007; Hemaiswarya et al., 2011). Cinnamaldehyde is capable of inhibiting the growth of *E. coli* O157:H7 and *Salmonella typhimurium* at concentrations similar to that of carvacrol and thymol; however, cinnamaldehyde is unable to disrupt the outer membrane or deplete the intracellular ATP pool (Helander et al., 1998). At a concentration of 2 μg/ml, Cinnamaldehyde was able to significantly decrease viability of *Helicobacter pylori* (Ali et al. 2005). Cinnamaldehyde has been shown to inhibit amino acid decarboxylases in *Enterobacter aerogenes*. The possible mechanism of action is thought to result from the carbonyl group binding to proteins, thus preventing the action of amino acid decarboxylases (Wendakoon and Sakauchi, 1995).

At sub lethal concentrations, cinnamaldehyde is capable of gaining access to the periplasm and inhibiting the activity of transmembrane ATPase (Hyldgaard et al., 2012). In a study conducted by Helander et al. (1998), trans-cinnamaldehyde was tested for its inhibitory effect against *E. coli* O157:H7 and *Salmonella typhimurium*. In addition, their toxicity to *Photobacterium leiognathi* was also investigated. It was determined that at 3mM, trans-cinnamaldehyde is capable of inhibiting *E. coli* and *Salmonella*. Interestingly, trans-cinnamaldehyde did not cause the outer membrane to become permeable but was able to inhibit enterobacterial growth and bioluminescence of *P. leiognathi*. It was concluded that trans-cinnamaldehyde is able to gain access to the periplasm as well as deeper parts of the bacterial cell. Gill and Holley (2006a,b) confirmed the ability of cinnamaldehyde to access the periplasm by demonstrating that increasing

concentrations of cinnamaldehyde (13.6-1362 μg/ml) can decrease ATPase activity of isolated cell membranes. Cell death, however, was not specifically attributed to ATPase inhibition because the concentration of cinnamaldehyde (681-1362 μg/ml) necessary to inhibit ATPase also resulted in membrane disruption of *E. coli* cells (Gill and Holley, 2006a).

While several studies have demonstrated that cinnamaldehyde interacts with the cell membrane, it is still quite unclear how membrane disruption occurs. Di Pasqua et al. (2007) observed that the general mechanism of action of cinnamaldehyde is not membrane disruption. When introduced to *E. coli*, *S. enterica*, *P. fluorescens*, and *B. thermosphacta*, cinnamaldehyde caused large increases in saturated fatty acids thereby altering the membrane lipid shape (Di Pasqua et al., 2006, 2007). The end result was a more rigid membrane and changed cell structure. In the case of *S. aureus*, the cell envelope disintegrated. When tested against fungi, the primary mechanism of action of cinnamaldehyde is thought to be cell division inhibition. Bang et al. (2000) demonstrated that in *Saccharomyces cerevisiae*, cinnamaldehyde inhibited cell wall synthesizing enzymes. Cinnamaldehyde acted as a non-competitive inhibitor of β-(1,3)-glucan synthase and a mixed inhibitor of chitin synthase isozymes.

As seen in geraniol, cinnamaldehyde is capable of acting synergistically against bacteria when combined with certain plant extracts. Moleyar and Narasimham (1992) demonstrated that a combination of cinnamaldehyde and eugenol at concentrations of 250 and 500 μg/ml, respectively, completely inhibited growth of *Staphylococcus* sp., *Micrococcus* sp. *Bacillus* sp. and *Enterobacter* sp. for more than 30 days. When the antimicrobials were tested individually, growth was not inhibited. Similarly, in a separate study, cinnamaldehyde combined with thymol or carvacrol inhibited growth of *Salmonella typhimurium* in Mueller Hinton broth (MHB) (Zhou et al., 2007).

The ability of essential oils to remain stable during food processing needs to be investigated. The heat stability of cinnamaldehyde was investigated. When heated alone, cinnamaldehyde was found to decompose to benzaldehyde at temperatures reaching 60 °C. When used in combination with eugenol or cinnamon leaf oil, cinnamaldehyde was heat stable, even when subjected to 200 °C for 30 minutes (Friedman et al., 2000). Cinnamaldehyde is an antimicrobial with great potential despite multiple studies debating its ability to disrupt bacterial cell membranes. More research is warranted in order to discover cinnamaldehyde's full potential as well as its true mechanism of action.

Salmonella enterica

Salmonella is a gram negative, non-spore-forming, rod shaped, facultative anaerobe that is part of the *Enterobacteriaceae* family (Hammack, 2012). *Salmonella* is split into two species: *Salmonella enterica* (*S. enterica*) and *Salmonella bongori*. *S. enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), and *S. enterica* subsp. *houtenae* (IV), *S. enterica* subsp. *indica* (VI) make up the six subspecies of *Salmonella enterica* (Hammack, 2012). Currently, over 2500 serovars belonging to the six subspecies of *Salmonella enterica* have been identified. The serovars of the subspecies are categorized based on flagellar, carbohydrate and lipopolysaccharide (LPS) structures (Coburn et al., 2007).

Common sources of contamination

Salmonella is vastly widespread in the environment and its existence is most likely attributed to various animal reservoirs of this organism. *Salmonella* is capable of colonizing the intestinal tracts of vertebrates, including humans, domestic pets, wildlife, and livestock

(Hammock, 2012). The only Salmonella serotypes that are found in humans are *Salmonella* typhi and *Salmonella* paratyphi (Miller and Pegues, 2005). Traditionally, *Salmonella* has been linked to poultry, eggs and dairy products, but more recently Salmonella has been linked to snakes, iguanas, frogs, pet tortoises, aquatic turtles, small turtles, pet bearded dragons, and pet crested geckos (CDC, 2015; Bouchrif et al., 2009; CDC, 2003; Mermin et al. 1997). Recently, more and more incidents of fresh fruits and vegetables such as cilantro, broccoli, cauliflower, and spinach serving as transmission sources of *Salmonella* have been documented (Bouchrif et al., 2009; Quiroz-Santiago et al., 2009). Fresh product contamination is mainly attributed to *Salmonella* entering through scar tissues, natural uptake via root systems, entrapment when produce are creating embryos, and transfer to edible plant tissues when produce are sliced.

Increased incidents of *Salmonella* contamination in fresh produce can be attributed to the increased importation of fresh produce from other countries. Fresh produce may become contaminated at any point during production, harvesting or processing. Many of these countries have climates that provide suitable growth temperatures for fresh fruits and vegetables all year. In addition, Good Agricultural Practices (GAPs) and Good Manufacturing Practices (GMPs) in many countries during production, harvesting, packaging, and distribution of fresh fruits and vegetables may not meet the minimum standards set forth by the United States. Hygienic conditions that are below minimum standards play a substantial role in microbial contamination of fresh produce. While poor hygienic conditions play an important role in microbial contamination, it is not the sole cause. Other factors such as using contaminated water to wash fresh produce, fertilizing crops with untreated sewage water, and improper handling of produce, all help facilitate the persistence of *Salmonella* on fresh fruits and vegetables.

Factors affecting growth and survival

Under various environmental conditions, *Salmonella* is about to propagate. For example, *Salmonella* does not have a requirement for sodium chloride, in order to multiply; however, in the presence of 0.4 to 4% sodium chloride, *Salmonella* is still able to grow. Most serotypes of Salmonella, optimally grow at temperatures ranging from 35 to 37°C, but can also grow between 5 to 47°C. If intrinsic (pH, water activity, oxidation-reduction potential) and extrinsic factors (gaseous atmosphere) are optimal, some *Salmonella* serotypes can even grow slowly at temperatures as high as 54°C or as low as 2 to 4°C (Gray and Fedorka-Cray, 2002). While *Salmonella* can grow in elevated temperatures, it is sensitive to heat and is easily destroyed at temperatures exceeding 70°C. At water activities (a_w) ranging between 0.99 and 0.94, Salmonella is able to grow. In dried food products, Salmonella is able to survive at a water activity of <0.2. Hanes (2003) and Bhunia (2008) observed that *Salmonella* can be completely inhibited at pH <3.8, water activity <0.94 or at temperatures <7 ºC.

Incidents of foodborne salmonellosis

Salmonella is responsible for approximately 1.1 million non-typhodial illnesses, 19,000 hospitalizations, and 378 deaths in the U.S. every year (CDC, 2011). One of the most prevalent Salmonella serotypes found predominately in developed countries is *Salmonella enterica* serotype Enteritidis. *Salmonella enteritidis*, was a major cause of foodborne human illiness, in the 1980s (Patrick et al. 2004). Based on the Centers for Disease Controls and Prevention's "Estimates for Foodborne Illness" (2011a), *Salmonella* was ranked one of the most problematic pathogens based on illnesses, hospitalizations, and deaths caused. Based on the assessment, *Salmonella* was responsible for an estimated 1,207,561 illnesses, 19,336 hospitalizations, and 378 deaths. Eggs

and raw or undercooked poultry are traditionally associated with *Salmonella* infections. This trend is slowly starting to change. In the recent years, *Salmonella* infections have been observed in foods ranging from fresh produce to fully processed foods. For example, from 2006 to 2015, various serovars of *Salmonella* were linked to contamination of various food products including: cucumbers, bean sprouts, nut butter, chia powder, raw cashew cheese, sesame paste, ground beef, mangoes, peanut butter, tomatoes, pot pies cantaloupes, cereals, pistachios, red and black pepper, alfalfa sprouts, Italian style meats, frozen fruit pulp, papayas, and ground tuna product (CDC, 2015). While many of these cases resulted in only a small amount of *Salmonella* infections there have been a few cases where a large population has been infected. In 2007, *Salmonella* serotype Tennessee was responsible for a multi-state outbreak involving peanut butter, which resulted in 425 infections from 44 states, 71 hospitalizations and zero deaths (CDC, 2007). In the same year, *Salmonella* I 4,[5],12:i:- ("four five twelve eye minus"), in pot pies, was responsible for causing 272 infections, 65 hospitalizations, and no deaths (CDC, 2007a). In 2008, jalapeno peppers, serrano peppers, and tomatoes, contaminated with *Salmonella* Saintpaul, were linked to causing 1442 infections, 286 hospitalizations, and 2 deaths (CDC, 2008). The following year, *Salmonella* Saintpaul, found in raw alfalfa sprouts, was responsible for 235 infections from 14 states, 7 hospitalizations and zero deaths (2009). Another significant outbreak in 2009 was credited to *Salmonella* Typhimurium. Peanut butter containing *Salmonella* Typhimurium was responsible for 714 infections in 46 states, 171 hospitalizations, and 9 deaths (CDC, 2009a). In 2010, *Salmonella* Montevideo in red and black pepper/Italian-style meats was responsible for 272 infections, 52 hospitalizations, and zero deaths (CDC, 2010). In the same year, *Salmonella* Enteritidis was linked to contaminated eggs in Iowa resulting in approximately 2000 illnesses reported from 11 states (CDC, 2010a). In 2012, both *Salmonella* Typhimurium and *Salmonella* Newport, in cantaloupes,

were responsible for 261 illnesses, 94 hospitalizations, and 3 deaths (CDC, 2012a). In addition, raw scraped ground tuna containing *Salmonella* Bareilly and *Salmonella* Nchanga was responsible for 425 illnesses, 55 hospitalizations and zero deaths (CDC, 2012). More recently, cucumbers have been a source of *Salmonella* infections. In 2014, *Salmonella* Newport in cucumbers was linked to 275 illnesses, 48 hospitalizations, and 1 death (CDC, 2014). Most recently, cucumbers, again, contaminated with *Salmonella* Poona has been linked to 732 illnesses, 150 hospitalizations, and 4 deaths (CDC, 2015a).

Characteristics of salmonellosis

The infection caused by *Salmonella* is known as salmonellosis. Salmonellosis can result in a variety of disease symptoms such as gastroenteritis, bacteremia, typhoid fever, and focal infections (Darwin and Miller, 1999). Gastroenteritis, as a result of *Salmonella*, typically resolves itself after a few days, but more severe cases especially those that are extremely young or quite elderly, immunocompromised individuals, or individuals with systemic infections may be subject to chemotherapy (Foley and Lynne, 2008; Lee et al., 2009). The population most susceptible to salmonellosis is the elderly, infants, and individuals with weak immune systems. When infected with *Salmonella*, a majority of individuals develop diarrhea, vomiting, abdominal cramps, and diarrhea 12 to 72 hours after ingesting an infected product (CDC, 2015). In addition, reactive arthritis, also known as Reiters syndrome, can develop after bacterial infections in certain individuals (Dworkin et al., 2001). The length of time in which Reiters syndrome can last is anywhere from several months to several years and typically causes swelling in feet, knees and ankles (Colville and Berryhill, 2007). No medical treatment beyond taking fluids orally is required for *Salmonella* infections. Generally, infections are resolved within 5 to 7 days. If the infection spreads from the intestines, ampicillin, trimethoprim-sulfamethoxazole, or ciprofloxacin will be

needed (CDC, 2015). While antibiotics have been shown to eliminate *Salmonella*, certain species demonstrate antibiotic resistance as a result of residing in animals that are fed antibiotics to help increase growth. Illnesses are not the only repercussions caused by salmonellosis. The annual cost of damages related to outbreaks of *Salmonella* in the United States has been estimated to be between $600 million and $3.6 billion dollars (Ter-Hsin et al., 2005).

Escherichia coli

Escherichia coli (*E. coli*) is a facultative anaerobic, gram negative, non-spore forming, rod-shaped bacteria. There are numerous *E. coli* strains present in environment and many of the strains do not pose significant danger. Interestingly some *E. coli* strains are beneficial to humans. *E. coli* is also known as an indicator organism, meaning if detected, in food and water, it is an indication of fecal contamination. *E. coli* can be separated into 6 distinct groups: enteropathogenic (EPEC), enterotoxigenic (ETEC), shiga-toxin producing/enterohemorrhagic (STEC/EHEC), enterinvasive (EIEC), enteroaggregative (EAEC), and diffusely adherent (DAEC) (Jafari et al., 2012). *E. coli* O157:H7 used in the experiments described in the present dissertation belong to group known as EHEC. The EHEC group is responsible for causing hemorrhagic colitis or other severe complication in infected individuals; therefore, the information in the proceeding paragraphs will place emphasis on *E. coli* O157:H7.

E. coli O157 is a foodborne pathogen well known throughout the world as it is commonly associated with foodborne disease outbreaks. The majority of *E. coli* associated foodborne outbreaks in the United States has been mostly attributed to the serotype O157:H7 (Karmali et al., 2010). In 1982, two outbreaks of hemorrhagic colitis led to the identification of *E. coli* O157:H7 as a human pathogen (Riley et al., 1983). In 1944, the H7 antigen was isolated from a human

diarrheal sample, whereas O157 was isolated from swine diarrheal samples and subsequently named in 1972 (Orskov et al., 1977).

Common sources of contamination

Generally speaking, *Escherichia coli* O157:H7 is transmitted via food and water. In several instances, transmission of *E. coli* O157:H7 has occurred through cross contamination. A substantial amount of outbreaks have been linked to food sources origination from cattle (i.e. ground beef and raw milk) (Pierard, 1992; Wall et al., 1996; Griffin, 1995). Steak that has been tenderized by injection, kabobs, salami, venison, ready to eat meats, jerky, butter, yogurt, milk, ice cream, coleslaw, grapes, apple juice, spinach, lettuce, alfalfa sprouts, and melons have all been implicated as hosts for *E. coli* O157:H7 (Rangel et al., 2005).

Factors affecting growth and survival

EHEC is capable of growing at temperatures ranging from 7 to 50 °C, with an optimum temperature of 37 °C. Certain strains of EHEC have the ability to grow in acidic foods having a pH as low as 4.4 (WHO, 2011). Specifically, E. coli O157:H7 grows optimally at temperatures between 30 to 42 °C, grows optimally between pH 6 to 8, and within 24 hours is unable to ferment sorbitol (Tsai and Ingham, 1997). EHEC can also grow in foods having a minimum water activity (a_w) of 0.95. To eliminate EHEC thermally, all parts of foods must reach a temperature of 70 °C or greater (WHO, 2011). Typically, *E. coli* takes residence among the normal microflora of the intestinal tract. Strains of *E. coli* that are considered harmful or pathogenic also reside in the intestinal tract. A defining characteristic of *E. coli* O157:H7 is its ability to release a shiga-like toxin that binds to endothelial cells thereby expressing globotriaosylceramide-3 (Gb3), which allows the toxin to be absorbed into the bloodstream and distributed to other organs. (Sandvig,

2001). Strains of *E. coli* that produce shiga toxins are commonly referred to as Shiga toxin producing E. coli (STEC) because the toxin produced bears resemblance to the toxin produced by Shigella dysenteriae type 1 (Calderwood et al., 1996). *E. coli* O157:H7 is closely related, to the enteropathogenic strain, *E. coli* O55:H7, renowned for causing diarrhea among infants across the world (Whittam et al., 1993). E. coli O157:H7, similar to enteropathogenic strains, can attach to epithelial cells and induce lesions (Nataro and Kaper, 1998).

Incidents of foodborne E. coli infections

The first documented *E. coli* O157:H7 foodborne outbreaks occurred in the United States in 1982 in Oregon and Michigan (CDC, 1982). As a result of ingesting the pathogen, individuals suffered from severe abdominal cramps and bloody diarrhea. A multistate outbreak occurred in fresh spinach in 2006. In this particular outbreak, across 26 states, *E. coli* O157:H7 caused 199 illnesses, 102 hospitalizations, 31 cases of hemolytic-uremic syndrome and 3 deaths (CDC, 2006). Another *E. coli* outbreak, in this case linked to Taco Bell, also occurred in 2006. A total of 71 illnesses were reported across 5 states. Among the 71 illnesses, 53 individuals were hospitalized and 8 developed kidney failure due to hemolytic uremic syndrome (CDC, 2006a). In 2009, ingestion of contaminated prepackaged cookie dough resulted in 72 cases of illness, 34 hospitalizations and 10 cases of hemolytic uremic syndrome (CDC, 2009b). Contaminated romaine lettuce, in 2011, resulted in 58 reported infections, 33 hospitalizations and 3 cases of hemolytic uremic syndrome (CDC, 2012b).

E. coli O157:H7 infections typically occur after ingestion of contaminated foods. The minimum infectious dose of *E. coli* O157:H7 ranges from 10 to 100 colony forming units and is drastically smaller in comparison to over one million colony forming units for other pathogenic E. coli strains (Greig et al., 2010). The human fatality rate as a result of E. coli infections is

approximately 1% (Besser et al., 1999). Once E. coli has been ingested it will begin to colonize the intestines and disease symptoms typically appear in 3 to 4 days. (Phillips et al., 2000). Initial symptoms begin with diarrhea and abdominal cramps, but eventually progress into bloody diarrhea (Mead and Griffin, 1998; Feng, 2012). As diarrhea becomes more severe, blood and mucus can be observed within the diarrhea at an occurrence of every 15 to 30 minutes (Feng, 2012). Hemolytic uremic syndrome can be developed in approximately 10-15% of individuals infected with E. coli O157:H7, 5-13 days after diarrhea occurs (Tarr et al., 2005; Scheiring et al., 2008). In more severe cases, patients may suffer seizures, coma, kidney failure, kidney damage or even death. (Martin et al., 1990; Bell et al., 1994; Boyce et al., 1995; MacDonald et al., 1998).

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CHAPTER 3.

ANTIMICROBIAL EFFICACY OF CINNAMALDEHYDE AGAINST *ESCHERICHIA COLI* O157:H7 AND *SALMONELLA ENTERIA* IN CARROT JUICE AND A MIXED BERRY JUICE HELD AT 4 °C OR 12 °C

A paper submitted to *Foodborne Pathogens and Disease*

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Running title: cinnamaldehyde kills pathogens in juices

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Abstract

The effectiveness of cinnamaldehyde for inactivating *Salmonella enterica* and *Escherichia coli* O157:H7 in carrot juice (CRJ) and a mixed berry juice (MBJ) was investigated. Brain heart infusion broth (BHI), CRJ and MBJ, with concentrations of added cinnamaldehyde ranging from 0.15 to 1.5 µl/mL, 0.25 to 2.0 µl/mL, and 0.25 to 1.5 µl/mL, respectively, were each inoculated with a 5-strain mixture of *S. enterica* or *E. coli* O157:H7 to give an initial viable count of 5.07 $\log_{10}$ CFU/mL. Inoculated BHI or juices without cinnamaldehyde served as control. Growth of the pathogens in BHI (35 ^{o}C) was monitored by taking absorbance readings (OD 600 nm) for 24 h. The inoculated juices were held at 4°C or 12°C for 24 h and numbers of viable pathogens were determined at 0, 2, 4, 8, and 24 h by plating samples on selective agar followed by incubation (35 ^{0}C) and counting bacterial colonies at 48 h. The minimum inhibitory concentration of cinnamaldehyde for both pathogens in BHI was 0.25 µl/mL. The pathogens were more sensitive to cinnamaldehyde in MBJ compared to CRJ irrespective of storage temperature ($P<0.05$). At 4 ^{o}C, cinnamaldehyde (1.5 µl/mL) completely inactivated *S. enterica* and *E. coli* in MBJ (negative by enrichment) within 2 h and 8 h, respectively, whereas, both pathogens were detected in CRJ (4 ^{o}C; with 2.0 µl/mL cinnamaldehyde) at 8 and 24 h. At 12 ^{o}C, *S. enterica* and *E. coli* were undetected in MBJ (1.5 µl/mL cinnamaldehyde) within 2 h and 4 h, respectively; however, in CRJ (12 ^{o}C; 2.0 µl/mL cinnamaldehyde), complete inactivation of *S. enterica* and *E. coli* occurred within 4 h and 24 h, respectively. Cinnamaldehyde is an effective antimicrobial from natural sources for inactivating bacterial pathogens in fruit and vegetable juices to enhance the microbial safety of these nutritious food products.

Introduction

Rising consumer demand for nutritious, low-calorie foods with fresh-like characteristics and heightened public awareness of the health benefits of consuming fresh produce have contributed to increased production and consumption of fruits, vegetables and fruit juices (Rico *et al*., 2007; Boeing *et al*., 2012). Many commercially available fruit and vegetable juices are either heat pasteurized, ultra-high-temperature processed or canned to ensure microbiological safety and extended shelf-life. While these processes are effective in destroying pathogenic microorganisms, they can negatively alter quality characteristics of juices such as color, flavor, and nutritional value (Mittal and Griffiths, 2005). In response to consumer demand for minimally processed foods with fresh-like attributes and growing public concern over perceived health risks posed by synthetic food preservatives, juice manufacturers continue to seek alternatives to traditional physical or chemical processes to enhance microbial safety of juices and newly developed juice blends. One potential alternative is the use of plant essential oils (EOs) and their isolated antimicrobial components (Davidson, 1997).

Plant EOs are aromatic, volatile extracts from various plant parts that have long been used for food preservation as well as for improving food flavor (Burt, 2004). Although the antimicrobial properties of EOs have been widely investigated, food applications for EOs have been limited by certain factors including variability in EO composition and antimicrobial activity, high concentrations of EO required for microbial control (Hyldgaard *et al*., 2012) and the undesirable alterations of food flavor from high levels of added EO (Yamazaki *et al*., 2004). One approach with potential to alleviate these challenges and allow better control over concentrations of natural antimicrobials added to foods is to use the extracted antimicrobial component(s) from EOs.

Cinnamaldehyde makes up approximately 1-8%, 55-75%, and 70-95% of the EO from cinnamon leaf, cinnamon bark, and cassia cinnamon bark, respectively (Ross, 1976). Cinnamaldehyde is generally recognized as safe (GRAS) by the U.S Food and Drug Administration and approved for food use (21 CFR 182.60). According to the U.S. Flavoring Extract Manufacturers Association, toxicity studies (sub chronic and chronic) revealed that cinnamaldehyde has a broad margin of safety (Adams *et al*., 2004). Although cinnamaldehyde has exhibited antimicrobial activity (Bilgrami *et al*., 1992; Burt 2004; Holley and Patel, 2005), its effectiveness in enhancing microbial safety of juices needs to be validated. Apart from inactivation of *E. coli* O157:H7 by cinnamaldehyde added to apple juice and apple cider (Baskaran *et al*., 2010), to our knowledge, there are no published reports on the antibacterial efficacy of cinnamaldehyde against enteric pathogens in juices such as carrot juice and mixed berry juice. Accordingly, the objective of the present study was to investigate the efficacy of low concentrations of cinnamaldehyde for inactivating *S. enterica* and *E. coli* O157:H7 in carrot juice and a mixed berry juice at refrigeration temperature (4°C) and at an abusive temperature (12°C).

Materials and Methods

Bacterial strains and culture conditions

Five serotypes of *Salmonella enterica* (Enteritidis-ATCC13076, Heidelberg, Typhimurium-ATCC 14802, Gaminara-8324, and Oranienburg-9329), and five strains of *Escherichia coli* O157:H7 (FRIK125, ATCC 35150, ATCC 43894, ATCC 43895, and 93-062) were obtained from the culture collection of the Microbial Food Safety Laboratory, Iowa State University, Ames, IA. Stock cultures were kept frozen (-70 ºC) in brain heart infusion (BHI) broth (Difco; Becton Dickinson, Sparks, Md) supplemented with 10% (vol/vol) glycerol. Frozen stock

cultures, thawed under cold running water, were activated in tryptic soy broth (TSB; pH 7.2; Difco, Becton Dickinson) at 35 °C. At least two consecutive 24-h transfers of each stock culture were performed before using the cells to inoculate BHI or juices for each experiment.

Preparation of inoculum

Equal volumes (6 mL per culture) of each of the working cultures of *S. enterica* and *E. coli* O157:H7 were combined in a sterile centrifuge tube. Cells were harvested by centrifugation (10,000 x g, 10 min, 4 °C) using a Sorvall Super T21 centrifuge (American Laboratory Trading, Inc., East Lyme, CT) and washed once in 0.85% (wt/vol) saline. Pelleted cells were suspended in fresh saline to obtain a final viable cell concentration of 9.0 $\log_{10}$ CFU/mL. Colony counts of the washed cell suspensions were evaluated by serially diluting (10-fold) and surface plating samples on tryptic soy agar (Difco, Becton Dickinson) supplemented with 0.6% yeast extract (TSAYE) followed by counting bacterial colonies on TSAYE after incubation (35 °C) for 24 h.

Preparation of treatment solutions for Bioscreen C assay

Certified foodgrade cinnamaldehyde (Sigma Aldrich, Milwaukee, WI) was added to BHI broth. Portions of BHI broth with added cinnamaldehyde (0, 0.15, 0.25, 0.5, 1.0, 1.25, and 1.5 µl/ml), and adjusted to pH 7.4 using 1M sodium hydroxide or 1M hydrochloric acid, were filter sterilized using 0.22 µm pore size filters (Fisher Scientific). The BHI broth samples (2.5 mL) with the added cinnamaldehyde including control (BHI with no cinnamaldehyde) in test tubes were each inoculated with 25 µl of diluted *S. enterica* or *E coli* O157:H7 cell suspensions to obtain a final cell concentration of 5.07 $\log_{10}$ CFU/mL.

Minimum inhibitory concentration (MIC)

Inoculated samples (200 µl each) of BHI broth (pH 7.4 ± 0.2) were added in triplicate to a 100-well microtiter plate for the Bioscreen C Turbidometer (Growth Curves USA Piscataway, NJ) and incubated at 35 °C for 24 h. Optical density (OD) measurements were taken at 600 nm every 30 min, with shaking of samples for 10 seconds prior to each measurement. Minimum inhibitory concentration (MIC) was the lowest cinnamaldehyde concentration that inhibited (< 0.05 OD unit increase) growth for 24 h in BHI broth (35 ºC).

Juice Preparation and inoculation

Commercially available pasteurized carrot juice (CRJ) and mixed berry juice (MBJ) with no added preservatives, and each juice from the same production lot, were purchased from a local grocery store. The juices were transported in a portable cooler to the laboratory and stored at 4 ^{0}C ± 0.2 ºC until used. The juices were tested for *Salmonella*, *E. coli* O157:H7, and other microflora respectively, by surface plating on Xylose lysine tergitol agar (XLT), Sorbitol MacConkey agar (SMA), and TSAYE. Specifically, 1.0-ml aliquots of each juice were added to 1.0-mL portions of sterile buffered peptone water (BPW; Becton Dickinson, Sparks, Md) at pH 7.2 ± 0.2. The entire 2.0-mL mixture was surface plated on 8 plates (0.25 mL per plate) of SMA or XLT. Bacterial colonies were counted after 48 h of incubation (35 ^{o}C). Additionally, 1.0-mL aliquots of each juice were added to tubes of sterile TSB (10 mL each) followed by incubation (35 ^{o}C) for 24 h. After incubation, looped samples of TSB were streak-plated on appropriate selective agar which was then incubated (35 ºC) and examined for bacterial colonies at 24 and 48 h.

A portion (400 mL) of each juice type was aseptically transferred into a separate sterile screw cap glass bottle and held at 4 ºC. Filter-sterilized cinnamaldehyde was added to each type

of juice to give the following concentrations: 0 (control), 0.25, 0.5, 1.5 and 2.0 µl/mL. Bottles of juice were capped, vigorously shaken and inoculated with 4.0 mL of a diluted (1:100 in 0.85% saline) cell suspension of *S. enterica* or *E. coli* O157:H7 to give a final cell concentration of 5.07 $\log_{10}$ CFU/mL for each pathogen. Each bottle of juice was swirled to mix its contents and stored at 4 ^{0}C or 12 ^{0}C.

Measurement of pH and Brix

Measurements of pH were taken using an Orion Model 525 pH meter (Orion Research, Inc., Boston, Massachusetts) fitted with a glass electrode. Before performing the pH measurements, all juice samples were tempered to 23 °C ± 1 °C. A digital Pocket Refractometer PAL (ATAGO, USA, Inc.) was used to take Brix measurements.

Microbiological analysis

Inoculated juices stored at 4 °C and 12 °C were tested for viable pathogens at 1, 2, 4, 8, and 24 h. Ten-fold serial dilutions of the juice were prepared using sterile BPW (pH 7.2). Aliquots (1.0 or 0.1 mL) of juice were surface plated (in duplicate) on XLT and SMA. Agar plates were incubated at 35 °C and bacterial colonies were counted at 48 h. All agar media were purchased from Difco, Becton Dickinson.

Presumptive *E. coli* O157:H7 or *S. enterica* colonies (~ 2 to 3 colonies per agar plate) were confirmed using biochemical test kits obtained from Scientific, Remel Products, Lenexa, KS. Confirmation of *E. coli* O157:H7 was performed by testing for the presence of O157 and H7 antigens using the *E. coli* O157:H7 agglutination test (RIM®, *E. coli* O157:H7 Latex Test Kit). The OXOID *Salmonella* Latex Test Kit was used to confirm presumptive *S. enterica* colonies.

The inoculated juices were enriched by aseptically transferring them to an enrichment broth and incubation (35 °C) for 48 h. Looped samples of enrichment broth were streak-plated on SMA and XLT and incubated (35 °C) for 24 h. Bacterial colonies were confirmed as *E. coli* O157:H7 or *S. enterica* as previously described.

Statistical analysis

Three replications of each experiment were performed. Mean numbers of pathogen survivors were analyzed using SAS statistical software version 9.3 (SAS Institute Inc., Cary, N.C.). Treatment means were evaluated for significant differences ($P<0.05$) using the Waller-Duncan test.

Results

Growth inhibition of pathogens by cinnamaldehyde in BHI broth

The effect of cinnamaldehyde (0, 0.15, 0.25, 0.5, 1.0, 1.25, and 1.5 µl/mL) on the growth of both pathogens in BHI broth (35 °C) at pH 7.4 is shown in figures 1 and 2. Cinnamaldehyde concentrations from 0.25 to 1.5 µl/mL completely inhibited growth of *S. enterica* and *E. coli* O157:H7; absolutely no increase in OD_{600nm} for any of the two pathogens was observed at concentrations higher than 0.15 µl/mL for 24 h (Figures 1 and 2). The MIC of cinnamaldehyde for both pathogens in BHI broth (35 °C) was 0.25 µl/ml.

Background microflora, juice characteristics, and initial viable count

No *Salmonella*, generic *E. coli* or *E. coli* O157:H7 was detected in non-inoculated CRJ or MBJ juice; however, there was a low level of background microflora in CRJ (1 to 3 CFU/mL) and MBJ (0 to 1 CFU/mL). The initial average pH of CRJ and MBJ was 6.25 and 3.59, respectively.

The initial °Brix values for the juices were 8.5 (for CRJ) and 12.3 (for MBJ). The addition of cinnamaldehyde did not significantly change the pH or °BRIX in any of the two juices (data not shown). The average initial viable count for *S. enterica* or *E. coli* O157:H7 in artificially inoculated control juice and juice with added cinnamaldehyde was 5.07 (± 0.2) Log_{10} CFU/mL.

Viability of pathogens in carrot juice at 4 °C and 12 °C

Viable counts of *S. enterica* after 24 h were 3.93 and 4.83 Log_{10} CFU/mL in CRJ held at 4 °C and 12 ºC, respectively (Table 1). At 4 ºC significant ($P<0.05$) reductions of the pathogen first occurred at 8 h and 24 h in juice containing 2.0 and 1.5 µl/mL, respectively, representing the two highest concentrations of cinnamaldehyde tested (Table 1A). At 12 ºC, significant ($P<0.05$) reductions occurred as early as 1 h in juice with cinnamaldehyde at 0.5, 1.5 or 2.0 µl/mL (Table 1B) indicating a greater sensitivity of the pathogen to cinnamaldehyde at the higher temperature (12 ºC). At 4 ºC, no complete inactivation (based on enrichment tests) of *S. enterica* occurred in carrot juice with any of the cinnamaldehyde concentrations evaluated (Table 1A). In contrast, at 12 ºC, cinnamaldehyde (2.0 µl/mL) completely inactivated *S. enterica* at 4, 8, and 24 h of storage (Table 1 B) based on results of selective plating and enrichment.

Increased pathogen sensitivity to cinnamaldehyde in carrot juice at 12 ºC compared to 4 ºC were also observed for *E. coli* O157:H7 in carrot juice (Table 2 A and B). At 24 h cinnamaldehyde (1.5 µl/mL) completely inactivated (negative enrichment test) *E. coli* O157:H7 in CRJ at 12 ºC whereas, at 4 ºC the pathogen was detected (positive enrichment) in juice containing that same concentration of cinnamaldehyde (Table 2 A and B).

Viability of pathogens in mixed berry juice at 4 °C and 12 °C

Viable counts of *S. enterica* after 1h of exposure to the control MBJ were 3.92 (at 4 °C) and 3.58 (at 12 °C) Log_{10} CFU/mL, representing 1.15 and 1.49 Log reduction, respectively (Table 3). At 4 °C and 12 °C, cinnamaldehyde (1.5 µl/mL) completely inactivated *S. enterica* in MBJ within 2 h; no salmonellae were detected by plating or enrichment. In contrast, at 2 h, viable salmonellae were 4.0 and 3.63 Log_{10} CFU/mL, in control MBJ at 4 ºC and 12 ºC, respectively.

Survival of *E. coli* O157:H7 in the more acidic MBJ was greater than that of *S. enterica* irrespective of storage temperature (Table 4). Reductions of *E. coli* O157:H7 after 1 h exposure to control MBJ at 4 °C and 12 °C were only 0.95 and 1.02 Log_{10} CFU/mL, respectively. In comparison to *S. enterica*, a longer time elapsed before complete inactivation of *E. coli* O157:H7 was observed in MBJ with cinnamaldehyde (1.5 µl/mL); *E. coli* O157:H7 was not detected (by plating or enrichment) within 8 h and 4 h in MBJ held at 4 °C and 12 °C, respectively ($P<0.05$).

Discussion

Several published reports describe the use of plant-based components including essential oils (Friedman et al., 2004), herb extract (Wu et al., 2008), caffeic acid (Reinders et al., 2001), cinnamon (Yutse and Fung, 2004), and cinnamaldehyde (Baskaran et al., 2010) for inactivating *E. coli* O157:H7 in apple juice or apple cider; however, the present study investigated the effectiveness of cinnamaldehyde for inactivating *S. enterica* and *E. coli* O157:H7 and in two types of fruit juices with substantially different pH and °Brix values. Those pathogens would typically be the most resistant organisms in juices (USDA, 2001).

The extent of inactivation of both pathogens in CRJ and MBJ increased with higher concentrations of cinnamaldehyde. For example, at 24 h, log_{10} CFU/mL reductions of viable *S.*

enterica in CRJ (4 ºC) with cinnamaldehyde at 0.25, 0.5, 1.5 and 2.0, were 1.13, 1.51, 3.60, and 4.48, respectively (Table 1A). At those same cinnamaldehyde concentrations in CRJ, $\log_{10}$ CFU/mL reductions of viable *E. coli* O157:H7 were 1.36, 1.87, 4.55, and 5.07, respectively (Table 2 A). Based on these results, cinnamaldehyde is a concentration-dependent antimicrobial that increases bacterial kill with increasing concentrations. In addition to cinnamaldehyde concentration, temperature was another factor that impacted the antibacterial activity of cinnamaldehyde in the juices.

In CRJ and MBJ held at 4 ºC or 12 ºC, cinnamaldehyde exhibited increased antimicrobial effectiveness at 12 ºC compared to 4 ºC. Our findings agree with reports on cinnamon or cinnamaldehyde in apple juice (Baskaran et al., 2010; Yutse and Fung, 2004; Zhao et al., 1993). For example, Baskaran et al. (2010) reported that at 4 ºC, viable *E. coli* O157:H7 in apple juice (4 ºC) with cinnamaldehyde (0.25 µl/mL) decreased to undetectable levels at 14 days; however, at 23 ºC the pathogen was undetectable at 5 days. Yutse and Fung (2004) observed that a combination of cinnamon and nisin caused greater inactivation of *Salmonella* Typhimurium and *E. coli* O157:H7 in apple juice at 20 ºC compared to 5 ºC. Mossel and de Bruin (1960) reported decreased viability of *Salmonella* Typhimurium and *E. coli* in apple juice at 24 ºC compared 5 ºC. Also, in apple cider without preservatives, the decline in viability of *E. coli* O157:H7 was greater at 25 ºC compared to 8 ºC (Zhao et al., 1993).

Enhanced antibacterial effect of cinnamaldehyde at higher temperatures is likely due to increased rate of cellular metabolism, growth, and death that occur at ambient temperature compared to refrigeration temperature (Levin and Rosen, 2006; Yutse and Fung, 2004). Bacterial sensitivity to certain antimicrobials may be attributed in part to increased fluidity of the cytoplasmic membrane that occurs at warmer temperatures (McElhaney, 1976). Stringent control

of membrane fluidity is crucial for membrane-associated functions such as active transport of solutes, passive permeability to hydrophobic molecules and protein-protein interactions (Zhang and Rock, 2008). The cytoplasmic membrane is the primary cellular site where cinnamaldehyde exerts its antibacterial action (Gill and Holley, 2006); therefore, cinnamaldehyde, by exerting a membrane-fluidizing effect (Di Pascua et al., 2006) may, at warmer temperatures, further disrupt those previously stated membrane-associated functions. Although the precise mechanism of antibacterial action of cinnamaldehyde is inconclusive, it is believed that cinnamaldehyde inactivates bacteria by inhibiting ATPase at sub-lethal concentrations and disrupting the cytoplasmic membrane at lethal concentrations (Hyldgaard *et al*., 2012).

Juice was held at 12 °C to simulate a temperature abused product that could result in growth of foodborne pathogens. The temperature of 12 °C is within the temperature danger zone (4.4 to 60 °C) in which enteric bacteria can grow and to numbers thereby increasing the risk of foodborne illness. At 4 °C and 12 °C cinnamaldehyde was effective in controlling both pathogens with a greater loss of pathogen viability in MBJ.

The intrinsic low pH (3.39) of MBJ could have also influenced the antibacterial activity of cinnamaldehyde by causing acid-induced sub-lethal injury in both pathogens thus presenting additional cellular lesions for the pathogens to cope with in the presence of cinnamaldehyde. Considering that the cytoplasmic membrane is a primary target for cinnamaldehyde (Gill and Holley, 2006) and that low pH can denature membrane proteins, the acidity of MBJ could sensitize the pathogens to the membrane damaging action of cinnamaldehyde. At sub-lethal concentrations, cinnamaldehyde inhibited ATPase activity in *E. coli* (Helander et al., 1998). In this regard, it was concluded that cinnamaldehyde crosses the outer membrane of Gram-negative bacteria, enters the

periplasm and possibly interacts with the cytoplasmic membrane (Helander et al., 1998) where the ATPases are located (Barton, 2005).

Conclusions

Cinnamaldehyde (ranging from 0.25 to 2.0 µl/mL) has good potential for killing *S. enterica* and *E. coli* O157:H7 in CRJ and MBJ at 4 ºC and at 12 ºC. Cinnamaldehyde concentrations (0.5 and 0.25 µl/mL or lower) in juices could potentially be used to enhance the antimicrobial effects of emerging food processing technologies such as high pressure processing, high pressure homogenization, ultra sound and pulse electric fields. Further research is needed in those areas as well as on effects of cinnamaldehyde concentrations on sensory characteristics of minimally processed juices.

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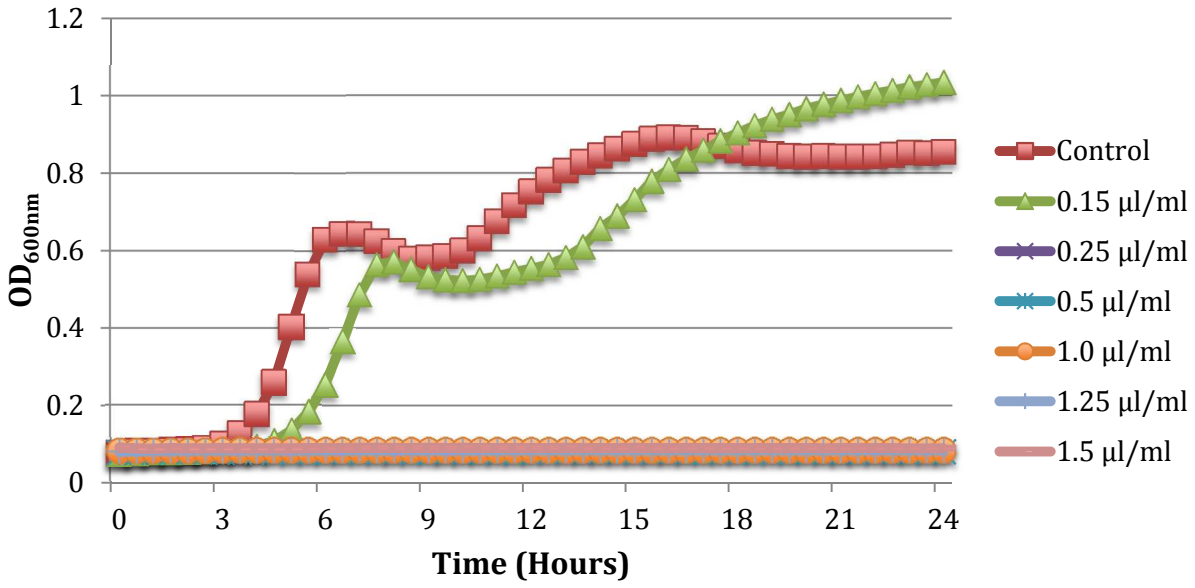

Figure 1. Survival and growth of *Salmonella enterica* in Brain heart infusion broth (pH 7.4; 35 °C) with various concentrations of added cinnamaldehyde.

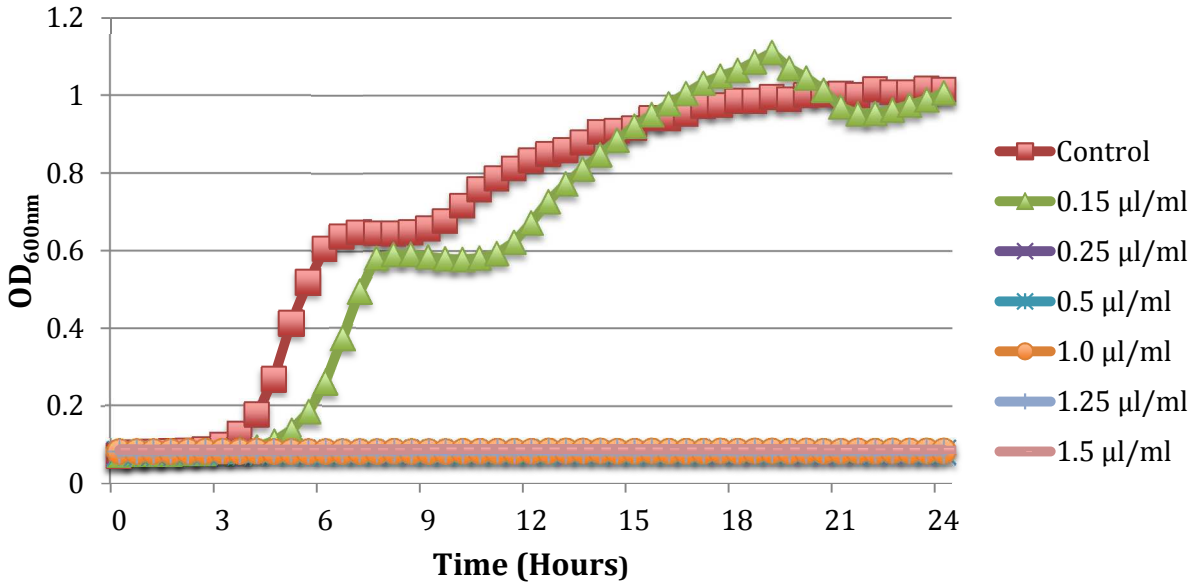

Figure 2. Survival and growth of *Escherichia coli* O157:H7 in Brain heart infusion broth (pH 7.4; 35 °C) with various concentrations of added cinnamaldehyde.

Table 1. Antibacterial effectiveness of cinnamaldehyde against *Salmonella enterica in carrot juice held at 4 °C (A) or 12 °C (B) for 24 hours.**

A	Viable count (Log_{10} CFU ml^{-1})[x] at 4 °C				
Treatment (µl mL^{-1})	1h	2h	4h	8h	24h
Control	4.45 ± 0.64a	4.21 ± 0.66a	4.09 ± 0.68a	4.40 ± 0.61a	3.93 ± 0.71a
Cinnamaldehyde (0.25)	4.12 ± 0.73a	4.40 ± 0.51a	4.06 ± 0.80a	4.39 ± 0.71a	3.94 ± 0.56a
Cinnamaldehyde (0.5)	4.15 ± 0.78a	4.13 ± 0.75a	4.21 ± 0.82a	4.27 ± 0.77a	3.56 ± 0.39a
Cinnamaldehyde (1.5)	4.17 ± 0.71a	4.10 ± 0.80a	3.83 ± 0.44a	3.40 ± 0.47ab	1.47 ± 1.31b
Cinnamaldehyde (2.0)	3.48 ± 0.36a	3.87 ± 0.39a	3.26 ± 0.30a	2.45 ± 0.32b	0.59 ± 1.03b
B	Viable count (Log_{10} CFU ml^{-1})[x] at 12 °C				
Treatment (µl mL^{-1})	1h	2h	4h	8h	24h
Control	4.14 ± 0.34a	4.24 ± 0.23a	4.20 ± 0.48a	4.39 ± 0.58a	4.83 ± 0.42a
Cinnamaldehyde (0.25)	3.79 ± 0.29ab	3.98 ± 0.11a	4.33 ± 0.46a	4.09 ± 0.42ab	3.06 ± 0.58b
Cinnamaldehyde (0.5)	3.65 ± 0.20bc	3.81 ± 0.17a	3.92 ± 0.30a	3.68 ± 0.21b	1.48 ± 0.27c
Cinnamaldehyde (1.5)	3.31 ± 0.19bc	2.83 ± 0.94b	1.54 ± 0.23b	0.62 ± 0.56c	0.16 ± 0.28d
Cinnamaldehyde (2.0)	3.28 ± 0.25c	2.72 ± 0.01b	ND; -ve	ND; -ve	ND; -ve

[x]Each value for viable count is the mean (standard deviation) of three replicate experiments

[a,b,c,d]Means with a different letter within a column differ significantly ($P<0.05$)

ND = no colonies detected on agar plates with the lowest dilution (1:3) of the sample

-ve = negative enrichment test

*Initial viable count of *S. enterica*: 5.04 ± 0.03 Log_{10} CFU ml^{-1}

Table 2. Antibacterial effectiveness of cinnamaldehyde against *E. coli* O157:H7* in carrot juice held at 4 °C (A) or 12 °C (B) for 24 hours.

A	Viable count (Log_{10} CFU ml^{-1})[x] at 4 °C				
Treatment (µl mL^{-1})	1h	2h	4h	8h	24h
Control	4.29 ± 0.20a	4.34 ± 0.26a	4.26 ± 0.19ab	4.04 ± 0.03a	3.75 ± 0.38a
Cinnamaldehyde (0.25)	4.08 ± 0.35a	4.31 ± 0.40a	4.48 ± 0.15a	3.73 ± 0.25a	3.71 ± 0.22a
Cinnamaldehyde (0.5)	4.26 ± 0.20a	4.25 ± 0.49a	4.29 ± 0.11a	3.67 ± 0.17a	3.20 ± 0.25b
Cinnamaldehyde (1.5)	4.14 ± 0.08a	4.23 ± 0.16a	3.87 ± 0.09bc	3.09 ± 0.37b	0.52 ± 0.45c
Cinnamaldehyde (2.0)	4.11 ± 0.16a	4.22 ± 0.33a	3.86 ± 0.36c	2.62 ± 0.44b	ND; +ve
B	Viable count (Log_{10} CFU ml^{-1})[x] at 12 °C				
Treatment (µl mL^{-1})	1h	2h	4h	8h	24h
Control	4.74 ± 0.02a	4.75 ± 0.08a	4.51 ± 0.43a	4.80 ± 0.30a	5.26 ± 0.43a
Cinnamaldehyde (0.25)	4.50 ± 0.11a	4.19 ± 0.30b	4.04 ± 0.60a	4.16 ± 0.44a	3.58 ± 0.29b
Cinnamaldehyde (0.5)	4.37 ± 0.27a	3.88 ± 0.19b	3.48 ± 0.64ab	3.98 ± 0.56a	2.68 ± 0.18c
Cinnamaldehyde (1.5)	3.59 ± 0.25b	3.73 ± 0.13bc	2.73 ± 0.89bc	1.10 ± 0.59b	ND; -ve
Cinnamaldehyde (2.0)	3.66 ± 0.33b	3.21 ± 0.54c	2.29 ± 0.31c	0.16 ± 0.28c	ND; -ve

[x]Each value for viable count is the mean (standard deviation) of three replicate experiments

[a,b,c,d]Means with a different letter within a column differ significantly ($P<0.05$)

ND = no colonies detected on agar plates with the lowest dilution (1:3) of the sample

-ve = negative enrichment test; +ve = positive enrichment test

*Initial viable count of *E. coli* O157:H7: 5.04 ± 0.02 Log_{10} CFU ml^{-1}

Table 3. Antibacterial effectiveness of cinnamaldehyde against *Salmonella enterica in mixed berry juice held at 4 ºC (A) or 12 ºC (B) for 24 hours**.

A	Viable count (Log_{10} CFU ml^{-1})[x] at 4 ºC				
Treatment (µl mL^{-1})	1h	2h	4h	8h	24h
Control	3.92 ± 0.62a	4.00 ± 0.34a	3.62 ± 0.07a	3.62 ± 0.19a	3.64 ± 0.02a
Cinnamaldehyde (0.25)	3.61 ± 0.13a	3.89 ± 0.26a	3.62 ± 0.18a	3.46 ± 0.43a	3.16 ± 0.21a
Cinnamaldehyde (0.5)	3.61 ± 0.34a	3.62 ± 0.17a	3.27 ± 0.22a	2.59 ± 0.46b	0.56 ± 0.97b
Cinnamaldehyde (1.5)	2.56 ± 1.20a	ND; -ve	ND; -ve	ND; -ve	ND; -ve
B	Viable count (Log_{10} CFU ml^{-1})[x] at 12 ºC				
Treatment (µl mL^{-1})	1h	2h	4h	8h	24h
Control	3.58 ± 0.09a	3.65 ± 0.31a	3.74 ± 0.15a	3.81 ± 0.28a	3.39 ± 0.26a
Cinnamaldehyde (0.25)	3.61 ± 0.14a	3.75 ± 0.23a	3.65 ± 0.29a	3.49 ± 0.14a	2.61 ± 0.05b
Cinnamaldehyde (0.5)	3.48 ± 0.13a	3.45 ± 0.53a	3.07 ± 0.10b	2.25 ± 0.30b	0.16 ± 0.28c
Cinnamaldehyde (1.5)	2.51 ± 0.54b	ND; -ve	ND; -ve	ND; -ve	ND; -ve

[x]Each value for viable count is the mean (standard deviation) of three replicate experiments

[a,b,c,d]Means with a different letter within a column differ significantly ($P<0.05$)

ND = no colonies detected on agar plates with the lowest dilution (1:3) of the sample

-ve = negative enrichment test; +ve = positive enrichment test

*Initial viable count of *S. enterica*: 5.04 ± 0.03 Log_{10} CFU ml^{-1}

Table 4. Antibacterial effectiveness of cinnamaldehyde against *E. coli* O157:H7* in mixed berry juice held at 4 ºC (A) or 12 ºC (B) for 24 hours.

A	Viable count (Log_{10} CFU ml^{-1})[x] at 4 ºC				
Treatment (µl mL^{-1})	1h	2h	4h	8h	24h
Control	4.12 ± 0.41a	4.11 ± 0.39a	3.99 ± 0.43a	3.86 ± 0.40a	3.65 ± 0.15a
Cinnamaldehyde (0.25)	4.00 ± 0.27a	4.08 ± 0.39a	3.93 ± 0.33a	3.71 ± 0.42a	3.38 ± 0.07ab
Cinnamaldehyde (0.5)	4.00 ± 0.32a	3.88 ± 0.20a	3.81 ± 0.30a	3.56 ± 0.15a	3.09 ± 0.47b
Cinnamaldehyde (1.5)	3.57 ± 0.27a	3.58 ± 0.81a	1.25 ± 1.21b	ND; -ve	ND: -ve
B	Viable count (Log_{10} CFU ml^{-1})[x] at 12 ºC				
Treatment (µl mL^{-1})	1h	2h	4h	8h	24h
Control	4.05 ± 0.02a	4.08 ± 0.15a	3.97 ± 0.13a	4.19 ± 0.24a	4.02 ± 0.28a
Cinnamaldehyde (0.25)	4.04 ± 0.07a	4.03 ± 0.11a	3.91 ± 0.02a	3.79 ± 0.06b	3.59 ± 0.08b
Cinnamaldehyde (0.5)	4.00 ± 0.05a	3.98 ± 0.10a	3.72 ± 0.14a	3.59 ± 0.10b	2.63 ± 0.19c
Cinnamaldehyde (1.5)	3.46 ± 0.12b	3.23 ± 0.29b	ND; -ve	ND; -ve	ND; -ve

[x]Each value for viable count is the mean (standard deviation) of three replicate experiments

[a,b,c,d]Means with a different letter within a column differ significantly ($P<0.05$)

ND = no colonies detected on the agar plates with the lowest dilution (1:3) of the sample

-ve = negative enrichment test; +ve = positive enrichment test

*Initial viable count of *E. coli* O157:H7: 5.04 ± 0.02 Log_{10} CFU ml^{-1}

CHAPTER 4.

INACTIVATION OF *ESCHERICHA COLI* O157:H7 AND *SALMONELLA ENTERICA* BY GERANIOL IN CARROT JUICE AND A MIXED BERRY JUICE AT 4 °C AND 12 °C

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Key words: *E. coli* O157:H7, *Salmonella*, geraniol, carrot juice, berry juice, inactivation

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Abstract

This study investigated the antimicrobial effect of geraniol (GER) on *Salmonella enterica* and *Escherichia coli* O157:H7 in carrot juice (CRJ; pH 6.25) and a mixed berry juice (MBJ; pH 3.59). Brain heart infusion broth (BHI; pH 7.4), CRJ and MBJ with or without (control) added GER were inoculated with a 5-strain mixture of *S. enterica* or *E. coli* O157:H7 to give an initial viable count of ~5.08 $\log_{10}$ CFU/ml. Concentrations of GER added to broth ranged from 0.15 to 1.5 µl/ml (BHI) and from 0.5 to 1.5 or 2.0 µl/ml in each of the two juices. Inoculated BHI or inoculated juices without GER served as control. Growth of the pathogens in BHI (35 ^{o}C) was evaluated by optical density (OD) measurements (at 600 nm) in a Bioscreen C turbidometer. Pathogen survivors in juices held at 4°C or 12°C were evaluated at 1, 2, 4, 8, and 24 hours by plating juice samples on selective agar and counting bacterial colonies after 48 h of incubation (35 ^{0}C). For each pathogen, the minimum inhibitory concentration (MIC) of GER in BHI was 0.5 µl/ml. Both pathogens were more sensitive to GER in MBJ than in CRJ. At all GER concentrations tested in CRJ, numbers viable pathogens were substantially decreased; however, both pathogens were not completely inactivated in that juice (at 4 ^{o}C or 12 ^{o}C) throughout 24 h. Within 1 h, *S. enterica* and *E. coli* O157:H7 were completely inactivated (negative by enrichment) in MBJ (4 ^{o}C or 12 ^{o}C) with added GER at1.0 µl/ml ($P < 0.05$). Based on these results, GER is a good potential to inactivate *S. enterica* and *E. coli* O157:H7 in acidic fruit juices such as MBJ and serve as an alternative to thermal pasteurization treatment.

Introduction

With increased production and consumption of fruit and vegetable juices several outbreaks of *Escherichia coli* O157:H7 and *Salmonella enterica* infections associated with juices such as fresh apple (*3, 7, 8, 14*) and orange juice (*9, 13, 21*) have occurred in the 1990s. Those outbreaks prompted the U.S. Food and Drug Administration (FDA) to develop regulations (21 Code of Federal Regulations 120; *35*) requiring that juice processors implement Hazard Analysis and Critical Control Point (HACCP) in the manufacture of all fruit/vegetable juices sold wholesale. Based on the juice HACCP regulations, juice processors must demonstrate a 5 Log CFU/ml reduction of the pertinent pathogen in each juice (*35*). As juice processors develop novel blends of fruit juice and vegetable juice they must have a validated 5-log reduction process for each product. While such a reduction could be easily achieved by heat pasteurization, heat-processed juices typically undergo negative sensory changes and loss of heat labile nutrients such as vitamin C. To circumvent those problems and meet consumers' desire for nutritious, minimally processed juices with fresh-like characteristics, juice processors are exploring alternative interventions including certain non-thermal processes (such as high pressure processing and pulsed electric fields) and/or naturally derived antimicrobials for inactivating pathogens in juices.

Natural antimicrobials of plant origin such as essential oils (EOs) or certain components of EOs have potent antimicrobial activity (*6, 25, 26*). Geraniol (GER) is a component of the EO from citronella oil (*22*), palmarosa oil (*15*), rose oil (*2*), ninde oil (*1*), and *Monarda fistulosa* (*30*). It is an acyclic monoterpene alcohol that has a rose-like odor and a taste characterized as sweet floral rose, citrus with fruity, waxy nuances (*5*). Geraniol is currently used as a flavoring substance and is generally recognized as safe (*36*). The antimicrobial efficacy of GER in laboratory growth media has been reported (*6, 11, 16*); however, there are few published reports on the antibacterial

effectiveness of geraniol against foodborne pathogens in fruit juices. In those reports substantial variations in bactericidal concentrations of GER are reported for juices based on juice types. For example, GER at 0.6 µl/ml produced 2.2 to 2.4 log reduction in numbers of viable *E. coli* O157:H7 ATCC43895 in strawberry juice almost immediately after the pathogen was exposed to that juice (32). Raybaudi-Massilia et al. (25) reported that a concentration of 2 µl/ml geraniol was sufficient to kill *Salmonella* Enteritidis, *E. coli* and *Listeria innocua* in apple and pear juices; however, a higher concentration (6 µl/ml) was required to inactivate those same microorganisms in melon juice and tryptone soy broth. Additionally, reports on differences in pathogen resistance to GER in juices are scarce. In a study by Friedman et al. (*17*), GER exhibited a greater antimicrobial activity against *Salmonella enterica* compared to *E. coli* O157:H7 in cloudy apple juice held at 37 °C for 30 min. In this context, the bactericidal activity (BA_{50}) of geraniol against those pathogens was 0.023 ± 0 (for *S. enterica*) and 0.057 ± 0.05 (for *E. coli* O157:H7), respectively. The BA_{50} is the percentage of an antimicrobial that kills 50% of the target pathogen under the test conditions (*17*).

Escherichia coli O157:H7 is a human enteric pathogen which is of major public health concern in North America, Europe, Asia and several other areas of the world. This organism has a low infectious dose and infected persons, especially immunocompromised individuals, may experience bloody diarrhea, hemolytic ureic syndrome and thrombotic thrombocytopenic purpura (4). The number of reported cases of *E. coli* O157:H7 foodborne infections are less that those caused by *Salmonella*; however, *E. coli* O157:H7 infections are associated with life-threatening symptoms and much higher hospitalization rates (19, 28). *Samonella enterica* is a well-known human enteric pathogen world-wide. Foodborne disease caused by this pathogen is usually self-limiting gastroenteritis; however, post-infection medical complications such as septicemia,

reactive arthritis or aortic aneurysms have been reported (*18, 31, 33*). Both of these pathogens have been implicated in outbreaks of enteric disease linked to the consumption of contaminated juices (*7, 8, 9, 37*).

The current widespread production of fresh juices and juice blends that are consumed without the application of a microbial kill step warrants the need for novel approaches to destroy juice-borne pathogens to ensure the microbial safety of these nutritious foods. To our knowledge there is no published research on the antibacterial efficacy of geraniol against human enteric pathogens in blends of fruit juices or vegetable juices. Accordingly, the objective of the present study was to evaluate the antimicrobial efficacy of geraniol against *S. enterica* and *E. coli O157:H7* in carrot juice and a mixed berry juice blend.

Materials and Methods

Bacterial cultures and culture conditions. Five serotypes of *Salmonella enterica* (Enteritidis-ATCC13076, Heidelberg, Typhimurium-ATCC 14802, Gaminara-8324, and Oranienburg-9329), and five strains of *Escherichia coli* O157:H7 (FRIK125, ATCC 35150, ATCC 43894, ATCC 43895, and 93-062) from the culture collection of the Microbial Food Safety Laboratory, Iowa State University, Ames, IA were used in the present study. Frozen (-70 ºC) stock cultures in brain heart infusion (BHI) broth (Difco, Becton Dickinson, Sparks, MD) supplemented with 10% (vol/vol) glycerol were thawed under cold running water, activated in tryptic soy broth (TSB; Difco, Becton Dickinson) at pH 7.2, and incubated at 35 °C. Two consecutive 18 to 24-h transfers of each stock culture were performed before using the cells for each experiment.

Preparation of inoculum. To prepare a 5-strain mixture of each pathogen, equal volumes (6 ml per culture) of *S. enterica* or *E. coli* O157:H7 working cultures were combined in a sterile

centrifuge tube. The cells were harvested by centrifugation (10,000 x g, 10 min, 4 °C) using a Sorvall Super T21 centrifuge (American Laboratory Trading, Inc., East Lyme, CT) and washed once in sterile 0.85% (wt/vol) saline. The pelleted cells were suspended in fresh 0.85% (wt/vol) saline to obtain a final viable cell concentration of ~10^9 CFU/ml. Viable colony counts of the washed cell suspensions were evaluated by serially diluting (10-fold) the cell suspensions and surface plating samples on tryptic soy agar (Difco, Becton Dickinson) supplemented with 0.6% yeast extract (TSAYE). The cell suspensions were used to inoculate BHI broth or fruit and vegetable juice blends.

Preparation of treatment solutions for Bioscreen C assay. The GER was purchased from Sigma-Aldrich (Milwaukee, Wisconsin) and added to BHI broth to obtain the following concentrations: 0.15, 0.25, 0.35, 0.5, 1.0, 1.25, and 1.5 µl/ml. The BHI broth was sterilized by filtration using 0.22 µm pore size filters (Fisher Scientific, Fair Lawn, NJ). Before filtration, the pH of the BHI was adjusted to 7.4 using 1M sodium hydroxide or 1M hydrochloric acid. Broth samples (2.5 ml) with the added antimicrobial and control broth (no added antimicrobial) in test tubes were each inoculated with 25 µl of diluted (1:100) *S. enterica* or *E. coli* O157:H7 cell suspension to obtain a final cell concentration of ~ 1.2 x 10^5 CFU/ml of sample.

Determination of Minimum Inhibitory Concentration. Inoculated samples (aliquots of 200 µl) were added in triplicate to the wells of a 100-well microtiter plate for the Bioscreen C Turbidometer (Growth Curves USA Piscataway, NJ), an automated microbial growth analyzer and incubator. The microtiter plates were incubated in the Bioscreen C at 35 °C for 24 h. Optical density (OD) measurements were taken at 600 nm every 30 min, with shaking of samples for 10 seconds prior to each OD reading. Minimum inhibitory concentration (MIC) was defined as the

lowest treatment concentration that completely inhibited (< 0.05 OD unit increase) microbial growth for 24 h in BHI broth at 35 ºC.

Preparation and inoculation of juices. The CRJ and MBJ with no added preservatives and each set of juices with the same production lot numbers, were purchased from a local grocery store in Ames, IA. Juices were stored in a walk-in cooler at 4 ^{0}C ± 1 ºC. The juices were vigorously shaken and 400 ml of each juice was aseptically transferred to sterile glass bottles. The bottles of juice were held at 4 ºC until they were inoculated with *E. coli* O157:H7 or *Salmonella*. The GER was added to each juice to give the following concentrations: 0.25, 0.5, 1.0, 1.5, and 2.0 µl/ml. Juices without added GER served as control. Bottles of juice were vigorously shaken and then inoculated with 4.0 ml of the suspension of the washed foodborne pathogens to give a final cell concentration of ~ 1.2 x 10^5 CFU/ml for each pathogen. After inoculation, each flask of juice was swirled to mix its contents and stored at 4 ^{0}C in a walk-in cooler or 12 ^{0}C in a thermostatically controlled incubator.

Measurement of pH and Brix. Measurements of pH and degrees Brix (ºBrix) were performed on non-inoculated juice on the day of inoculation. The pH measurements were taken using an Orion Model 525 pH meter (Orion Research, Inc., Boston, Massachusetts) fitted with a glass electrode. Measurements of ºBrix were performed using a digital Pocket Refractometer PAL (ATAGO, USA, Inc.). All juice samples were tempered to 23 °C ± 1 °C before their pH and ºBrix were determined.

Microbial analysis. Inoculated juices stored at 4 °C and 12 °C were analyzed for numbers of viable pathogens at 1, 2, 4, 8, and 24 h. Ten-fold serial dilutions of the juice were prepared using sterile buffered peptone water (BPW; Becton Dickinson) at a pH of 7.2 ± 0.2. Aliquots (1.0 or 0.1

mL) of juice or diluted samples of juice were surface plated (in duplicate) on xylose lysine tergitol agar (XLT) and sorbitol MacKonkey agar (SMA). Agar plates were incubated at 35 °C and bacterial colonies were counted at 48 h. Biochemical test kits were used to confirm presumptive *E. coli* O157:H7 or *S. enterica* colonies (~ 2 to 3 colonies per agar plate). Confirmation of *E. coli* O157:H7 was performed by testing for O157 and H7 antigens using the *E. coli* O157:H7 agglutination test (RIM®, *E. coli* O157:H7 Latex Test Kit, Fisher Scientific, Remel Products, Lenexa, KS). Confirmation of presumptive *S. enterica* colonies was performed using the OXOID *Salmonella* Latex Test Kit (Fisher Scientific).

The inoculated juices were routinely enriched to cater for instances when no bacterial colonies were observed on any of the agar plates. The inoculated juices were enriched by aseptically transferring them to non-selective enrichment broth medium. For detection of *E. coli* O157:H7 and *S. enterica*, inoculated juice samples were enriched in TSB supplemented with 0.6% (w/v) yeast extract (YE; Difco) and Universal Pre-enrichment Broth (UPB; Sigma Aldrich), respectively. The inoculated broth in large pyrex tubes (23 by 140 mm) was incubated at 35 °C for 48 h. After incubation, samples of broth were streak-plated on SMA and XLT followed by incubation of the agar plates at 35 °C for 24 h before checking them for bacterial colonies. Selected bacterial colonies were confirmed as *E. coli* O157:H7 or *S. enterica* as previously described. Each enrichment test was performed in triplicate.

Data handling and statistical analysis. Three replications of each experiment were performed. Mean numbers of viable *S. enterica* and *E. coli* survivors were expressed as log CFU/ml. Log reduction of survivors for a pathogen in each juice was calculated by subtracting the numbers of survivors (log CFU/ml) at a selected exposure time, from the initial viable count of that pathogen (log CFU/ml). Mean numbers of survivors were statistically analyzed using SAS

statistical software version 9.3 (SAS Institute Inc., Cary, N.C.). Treatment means were evaluated for statistically significant differences using the Waller-Duncan test. Significant differences were defined at $P < 0.05$ for all the experimental data.

Results

Viability of pathogens in BHI broth. Figures 1 and 2 show the effect of GER on the growth of *E. coli* O157:H7 and *S. enterica*, respectively, in brain heart infusion (BHI) broth (pH 7.40). In the control broth *E. coli* O157:H7 and *S. enterica* and grew and reached optical densities (OD 600_{nm}) values of 0.64 and 0.66, respectively, at 8 h. Both pathogens grew in BHI containing concentrations of GER concentrations ranging from 0.15 to 0.35 µl/ml. Concentrations of GER at 0.5 µl/ml and higher prevented growth of both pathogens. The minimum inhibitory concentration (MIC) of GER for both *S. enterica* and *E. coli* O157:H7 in BHI broth (35 °C) was 0.5 µl/ml.

Natural microflora, juice characteristics, and initial viable count. *Salmonella*, generic *E. coli* or *E. coli* O157:H7 was not detected in the CRJ or MBJ juice by direct plating or enrichment of non-inoculated juices without added GER. A low level of background microflora in CRJ (1 to 3 CFU/ml) and MBJ (0 to 1 CFU/ml) was detected based on aerobic plate count of the non-inoculated juices. The initial average pH of CRJ and MBJ was 6.25 and 3.59, respectively. The initial °Brix values for the juices were 8.5 (for CRJ) and 12.3 (for MBJ). The addition of GER did not change the pH or °BRIX in any of the two juices. The average initial viable count for *S. enterica* or *E. coli* O157:H7 in artificially inoculated control juice and juice with added GER was 5.08 (± 0.2) $\log_{10}$ CFU/ml.

Viability of pathogens in carrot juice at 4 °C and 12 °C. Table 1 shows the effect of GER on the viability of *S. enterica* in CRJ (at 4 °C and 12 °C). In control CRJ (no added GER) *S.*

enterica survived well; numbers of survivors at 24 h were 3.93 and 4.83 $\log_{10}$ CFU/ml in juice held at 4 °C and 12 °C, respectively. At 24 h, GER at 1.0, 1.5, and 2.0 µl/ml decreased the numbers of viable *S. enterica* by 1.87, 2.72 and 4.56 log cycles, respectively, in CRJ at 4 °C. At those same GER levels, increased inactivation of the pathogen was observed at 24 h in juice held at 12 °C; log reductions of the pathogen were 2.64, 4.66, and 4.92. A similar trend was observed at 24 h for *E. coli* O157:H7 in CRJ (Table 2). At 1.0, 1.5, and 2.0 µl/ml GER decreased the initial numbers of viable *E. coli* O157:H7 by 1.81, 2.27 and 4.25 (CRJ at 4 °C) and by 2.01, 4.40, and 4.48 (CRJ at 12 °C). While GER produced substantial losses in viability of both pathogens, no complete inactivation (5.08 log reduction) was achieved in CRJ.

Viability of pathogens in mixed berry juice at 4 °C and 12 °C. Table 3 shows the antibacterial effect of GER against *S. enterica* in MBJ (at 4 °C and 12 °C). Some loss of viability of *S. enterica* occurred in control MBJ; viable counts of the pathogen after 1h of in MBJ were 3.92 (at 4 °C) and 3.58 (at 12 °C) $\log_{10}$ CFU/ml, representing 1.15 and 1.49 log reduction, respectively. A concentration of GER as low as 0.5 µl/ml exhibited strong bactericidal activity against *S. enterica* within 1 h (at 4 °C) and 2 h (at 12 °C) in MBJ; at those times no *Salmonella* colonies was observed based on selective plating of juice; however, non-selective enrichment tests were positive. For GER concentrations of 1.0 and 1.5 µl/ml, no *Salmonella* was detected (5.08 log reduction) by plating or enrichment of juice samples taken from 1 h through 24 h irrespective of the temperature at which the juice was held.

The effect of GER on numbers of viable *E. coli* O157:H7 in MBJ held at 4 °C and 12 °C is shown in Table 4. Compared to *S. enterica*, *E. coli* O157:H7 was more resistant to the GER (0.5 µl/ml) in MBJ. At both 4 °C and 12 °C, *E. coli* O157:H7 was detected up to 2 h by plating; however, at 4, 8, and 24 h the pathogen was detected only by enrichment. At 1.0 or 1.5 µl/ml GER, *E. coli*

O157:H7 was completely inactivated (5.08 log reduction) in MBJ (negative enrichment test) from 1 h through 24 irrespective of temperatures at which the juice samples were held.

Discussion

Essential oils (EOs) are typically products of the aromatic plant's secondary metabolism and are found in leaves, bark, and fruit (*20*). The major antimicrobial components of EOs are phenols, aldehydes, and terpenes (*10*) and monoterpene alcohols such as linalool, nerol, citronellol, and geraniol are known to have antibacterial and antifungal activity (*34*). EOs and some of their antimicrobial components exhibit bactericidal activity by disrupting the cytoplasmic membrane (*6*) to destroy vital cell functions including energy generation and nutrient transport (*27*). Geraniol is an acyclic monoterpene alcohol whose in vitro bactericidal action has been reported (*12, 29, 38*). Among 66 EOs evaluated for antimicrobial activity, geraniol was inhibitory to growth of human and animal pathogens including *Salmonella* Typhmurium and *Escherichia coli* (*29*). Results of published research have described the antibacterial effectiveness of EOs or selected EO components for inactivating pathogens such as *E. coli* O157:H7 in apple juice (*17*).

In the present study, GER inhibited growth of *S. enterica* and *E. coli* O157:H7 in laboratory broth (BHI) and inactivated those pathogens in juices (CRJ and MBJ). For example, at 24 h, log CFU/ml reductions in numbers of viable *S. enterica* in CRJ (4 ºC) with GER at 0.5, 1.0, 1.5 and 2.0 µl/ml, were 1.04, 1.87, 2.72 and 4.56, respectively (Table 1A). At those same concentrations of GER, log CFU/ml reductions of viable *E. coli* O157:H7 in CRJ (4 °C) were 1.41, 1.81, 2.27, and 4.25, respectively (Table 2 A). Based on these results GER inactivated *S. enterica* and *E. coli* O157:H7 in a dose-dependent manner in CRJ. In addition to GER concentration, juice pH was another factor that influenced the antibacterial activity of GER.

Within 1 h in the control MBJ (no added GER) at 4 °C the initial viable count (Log CFU/ml) of the pathogens decreased from 5.08 to 3.92 (*S. enterica*) and to 4.12 (*E. coli* O157:7) representing log reductions of 1.16 and 0.96, respectively. The low pH (3.59) of MBJ might have caused sub-lethal injury or death in part of the initial cell population of both pathogens. Since the presence of natural microflora in the juices precluded us from using non-selective agar to enumerate survivors, we accounted for non-injured survivors (cells that grew on the selective agar medium) and not sub-lethally injured cells that are unable to form colonies on selective agar (*23, 24*). However, in instances when a juice samples yielded no colonies on selective agar, our non-selective enrichment tests, when negative, provided evidence of complete inactivation of the target pathogen. Pertinent examples are presented in Tables 3 and 4 where GER at 1.0 and 1.5 µl/ml in MBJ resulted in no colonies of *S. enterica* or *E. coli* O157:H7 on selective agar media (from sampling MBJ at 1 h through 24 h) while none of the two pathogens could be detected by non-selective enrichment. In this instance both pathogens were presumed to be killed. Considering the fact that the initial viable count of each pathogen in the MBJ was 5.08 log CFU/ml, GER at 1.0 and 1.5 µl/ml met the 5.0 log reduction requirement of the juice HACCP regulations for ensuring the microbial safety of juices (*35*).

Both of those organisms used in the present study are human enteric pathogens that are frequently implicated in juice-related disease outbreaks. In fact, for reported outbreaks linked to fruit juices, *E. coli* O157:H7 and toxigenic *E. coli* strains are the most commonly implicated followed closely by *Salmonella* spp. (*37*). With the increasing production and consumption of fresh fruit and vegetable juices and novel juice blends that are minimally processed, applications of EOs or their components to destroy enteric pathogens in these “new” juices needs to be validated.

Results of this study indicate that concentrations GER (1.0 and 1.5 µl/ml) can completely inactivate *S. enterica* and *E. coli* O157:H7 to improve the microbial safety of MBJ at refrigeration temperature (4 ^{o}C) and under temperature abuse (12 ^{o}C). The far greater extent of pathogen inactivation in MBJ compared to CRJ suggests that judicious application of natural antimicrobials to juices that already have intrinsic antimicrobial properties such as low pH is necessary to achieve a cumulative 5-log reduction of the pertinent pathogen and avoid the application of thermal treatment. Further research is needed to evaluate the effects of GER on sensory characteristics of minimally processed fruit and vegetable juices.

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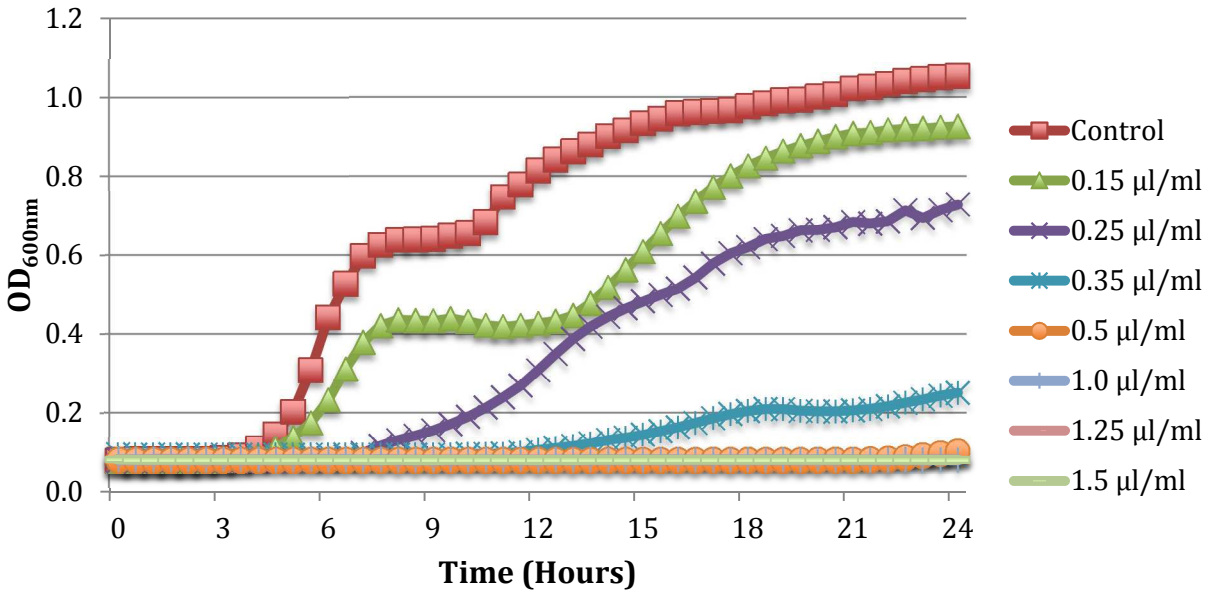

Figure 1. Growth of *E. coli* O157:H7 in BHI broth (35 °C) at pH 7.4 supplemented with various concentrations of geraniol.

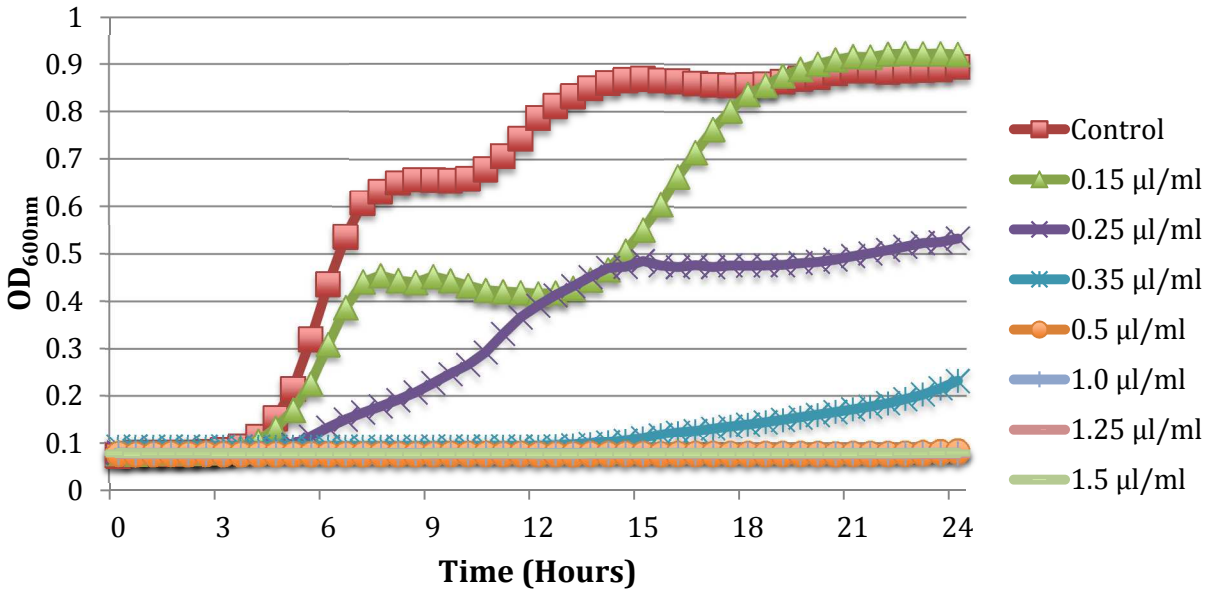

Figure 2. Growth of *Salmonella* in BHI broth (35 °C) at pH 7.4 supplemented with various concentrations of geraniol.

Table 1. Antibacterial effectiveness of geraniol against *Salmonella enterica* in carrot juice held at 4 ºC (A) or 12 °C (B) for 24 hours.

A	Viable count (Log_{10} CFU ml^{-1})[x]				
Treatment (mg ml^{-1})	1h	2h	4h	8h	24h
Control	4.45 ± 0.64a	4.21 ± 0.66a	4.09 ± 0.68a	4.40 ± 0.61a	3.93 ± 0.71a
Geraniol (0.5)	4.44 ± 0.66a	4.20 ± 0.69a	4.14 ± 0.64a	4.21 ± 0.38ab	4.04 ± 0.23a
Geraniol (1.0)	4.22 ± 0.56a	4.24 ± 0.67a	3.69 ± 0.74ab	4.06 ± 0.59ab	3.21 ± 0.32ab
Geraniol (1.5)	4.34 ± 0.64a	4.09 ± 0.67a	3.75 ± 0.35ab	3.48 ± 0.40bc	2.36 ± 0.76b
Geraniol (2.0)	4.05 ± 0.23a	3.15 ± 0.12a	2.93 ± 0.35b	2.74 ± 0.13c	0.52 ± 0.45c
B	Viable count (Log_{10} CFU ml^{-1})[x]				
Treatment (mg ml^{-1})	1h	2h	4h	8h	24h
Control	4.14 ± 0.34a	4.24 ± 0.23abc	4.20 ± 0.48a	4.39 ± 0.58a	4.83 ± 0.42a
Geraniol (0.5)	4.45 ± 0.22a	4.47 ± 0.42a	4.52 ± 0.06a	4.55 ± 0.31a	4.83 ± 0.13a
Geraniol (1.0)	4.46 ± 0.06a	4.44 ± 0.31ab	4.07 ± 0.36a	3.78 ± 0.22a	2.44 ± 0.40b
Geraniol (1.5)	4.11 ± 0.03a	3.94 ± 0.16bc	3.32 ± 0.32b	2.52 ± 0.17b	0.42 ± 0.39c
Geraniol (2.0)	4.21 ± 0.26a	3.77 ± 0.09c	2.94 ± 0.36b	1.88 ± 0.62b	0.16 ± 0.28c

[x]Each reported value for viable count represents the mean (standard deviation) of three replications of the experiment

[a,b,c]Means with a different letter within a column differ significantly ($P<0.05$)

Initial viable count of *S. enterica*: ~5.08 log_{10} CFU/ml

Table 2. Antibacterial effectiveness of geraniol against *E. coli* O157:H7 in carrot juice held at 4 ºC (A) and 12 °C (B) for 24 hours.

A	Viable count (Log_{10} CFU ml^{-1})[x]				
Treatment (mg ml^{-1})	1h	2h	4h	8h	24h
Control	4.29 ± 0.20a	4.34 ± 0.26ab	4.26 ± 0.19a	4.04 ± 0.03ab	3.75 ± 0.38a
Geraniol (0.5)	4.35 ± 0.21a	4.64 ± 0.36a	4.54 ± 0.49a	4.26 ± 0.16a	3.67 ± 0.61a
Geraniol (1.0)	4.28 ± 0.25a	3.70 ± 0.76b	4.39 ± 0.25a	3.83 ± 0.85ab	3.27 ± 0.52a
Geraniol (1.5)	4.35 ± 0.09a	4.53 ± 0.46ab	4.01 ± 0.24ab	3.41 ± 0.12b	2.81 ± 0.41a
Geraniol (2.0)	4.19 ± 0.31a	4.08 ± 0.21ab	3.62 ± 0.17b	2.54 ± 0.35c	0.83 ± 0.73b

B	Viable count (Log_{10} CFU ml^{-1})[x]				
Treatment (mg ml^{-1})	1h	2h	4h	8h	24h
Control	4.74 ± 0.02a	4.75 ± 0.08a	4.51 ± 0.43a	4.80 ± 0.30a	5.26 ± 0.43a
Geraniol (0.5)	4.52 ± 0.05ab	4.52 ± 0.23ab	4.73 ± 0.06a	4.72 ± 0.07a	5.03 ± 0.43a
Geraniol (1.0)	4.45 ± 0.17ab	4.30 ± 0.23b	3.77 ± 0.11b	3.77 ± 0.25a	3.07 ± 0.31b
Geraniol (1.5)	4.10 ± 0.42b	3.81 ± 0.15c	3.21 ± 0.54bc	2.31 ± 0.48c	0.68 ± 0.64c
Geraniol (2.0)	4.12 ± 0.48b	3.71 ± 0.30c	2.83 ± 0.41c	1.78 ± 0.94c	0.60 ± 0.67c

[x]Each reported value for viable count represents the mean (standard deviation) of three replications of the experiment

[a,b,c,d]Means with a different letter within a column differ significantly ($P<0.05$)

Initial viable count of *E. coli O157:H7*: ~5.08 log_{10} CFU/ml

Table 3. Antibacterial effectiveness of geraniol against *Salmonella enterica* in berry juice held at 4 ºC (A) and 12 °C (B) for 24 hours.

A	Viable count (log_{10} CFU/ml)[x]				
Treatment (µl/ml)	1h	2h	4h	8h	24h
Control	3.92 ± 0.62a	4.00 ± 0.34a	3.62 ± 0.07a	3.62 ± 0.19a	3.64 ± 0.02a
Geraniol (0.25)	3.67 ± 0.24a	3.62 ± 0.27a	3.20 ± 0.02a	3.18 ± 0.64a	2.07 ± 0.35b
Geraniol (0.5)	ND; +ve[y]	ND; +ve	ND; +ve	ND; +ve	ND; +ve
Geraniol (1.0)	ND; -ve	ND; -ve	ND; -ve	ND; -ve	ND; -ve
Geraniol (1.5)	ND; -ve	ND; -ve	ND; -ve	ND; -ve	ND; -ve

B	Viable count (log_{10} CFU/ml)[x]				
Treatment (mg ml^{-1})	1h	2h	4h	8h	24h
Control	3.58 ± 0.09a	3.65 ± 0.31a	3.74 ± 0.15a	3.81 ± 0.28a	3.39 ± 0.26a
Geraniol (0.25)	3.45 ± 0.13a	3.35 ± 0.39a	2.84 ± 0.16b	2.39 ± 0.09b	1.59 ± 0.26b
Geraniol (0.5)	1.05 ± 0.98b	ND; +ve	ND; +ve	ND; +ve	ND; +ve
Geraniol (1.0)	ND; -ve	ND; -ve	ND; -ve	ND; -ve	ND; -ve
Geraniol (1.5)	ND; -ve	ND; -ve	ND; -ve	ND; -ve	ND; -ve

[x]Each reported value for viable count represents the mean (standard deviation) of three replications of the experiment

[a,b,c]Means with a different letter within a column differ significantly ($P<0.05$)

[y]ND = no colonies detected on agar plates; enrichment test: -ve = negative or +ve = positive

Initial viable count of *S. enterica*: ~5.08 log_{10} CFU/ml

Table 4. Antibacterial effectiveness of geraniol against *E. coli* O157:H7 in berry juice held at 4 ºC (A) and 12 °C (B) for 24 hours.

A	Viable count (log_{10} CFU/ml)[x]				
Treatment (µl/ml)	1h	2h	4h	8h	24h
Control	4.12 ± 0.41a	4.11 ± 0.39a	3.99a ± 0.43a	3.86 ± 0.40a	3.65 ± 0.15a
Geraniol (0.5)	3.02 ± 0.56b	2.14 ± 1.44b	ND; +ve	ND; +ve	ND; +ve
Geraniol (1.0)	ND; -ve[y]	ND; -ve	ND; -ve	ND; -ve	ND; -ve
Geraniol (1.5)	ND; -ve	ND; -ve	ND; -ve	ND; -ve	ND; -ve

B	Viable count (log_{10} CFU/ml)[x]				
Treatment (µl/ml)	1h	2h	4h	8h	24h
Control	4.05 ± 0.02a	4.08 ± 0.15a	3.97 ± 0.13a	4.19 ± 0.24a	4.02 ± 0.28a
Geraniol (0.5)	2.71 ± 0.81b	2.01 ± 1.74b	ND; +ve	ND; +ve	ND; +ve
Geraniol (1.0)	ND; -ve	ND; -ve	ND; -ve	ND; -ve	ND; -ve
Geraniol (1.5)	ND; -ve	ND; -ve	ND; -ve	ND; -ve	ND; -ve

[x]Each value for viable count is the mean (standard deviation) of three replicate experiments

[a,b,c]Means with a different letter within a column differ significantly ($P<0.05$)

[y]ND = no colonies detected on agar plates; enrichment test: -ve = negative or +ve = positive

Initial viable count of *E. coli* O157:H7: ~5.08 log_{10} CFU/ml

CHAPTER 5.

INFLUENCE OF CINNAMALDEHYDE ON INACTIVATION OF *ESCHERICHIA COLI* O157:H7 AND *SALMONELLA ENTERICA* IN CARROT AND BERRY JUICES BY HIGH PRESSURE PROCESSING

A paper to be submitted to the Journal of Food Protection

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Abstract

The influence of cinnamaldehyde (CA) combined with high pressure processing (HPP) to inactivate *Escherichia coli* O157:H7 and *Salmonella enterica* in carrot juice (CRJ; pH 6.25) and a mixed berry juice (MBJ; pH 3.59) was investigated. Both juices each with added CA at concentrations of 0 (control), 0.10, 0.15 or 0.25 µl/ml were each inoculated with *E. coli* O157:H7 or *S. enterica* to obtain approximately 10^7 CFU/ml. The inoculated juices were pressurized at 300 or 400 MPa for 60, 90, or 120 seconds at 4 °C (initial temperature). Numbers of viable cells of each pathogen in the juices were evaluated before pressurization, after pressurization (within 1 h) and at set time intervals during refrigerated storage (4 °C) of the juices for 75 days. Both pathogens survived for as long as 42 days or more in control CRJ (no added CA) following HPP (400 MPa) for 60, 90, or 120 s. Addition of CA to juices increased the sensitivity of the pathogens to HPP with *S. enterica* exhibiting a greater loss in viability than *E. coli* O157:H7 to the CA/HPP combinations tested. CA (0.25 µl/ml) combined with 400 MPa (60 s) inactivated *Salmonella* by more than 5.5-log cycles in CRJ, whereas *E. coli* O157:H7 was inactivated by only 2.26 log cycles ($P<0.05$). For CRJ, the CA/HPP treatments tested were inadequate to produce an immediate 5-log reduction of *E. coli* O157:H7. In the more acidic MBJ, a treatment of CA (0.15 µl/ml) with a lower pressure (300 MPa for 120 s) resulted in complete inactivation (negative enrichment) and greater than a 5-$\log_{10}$ CFU/ml reduction of both *E. coli* O157:H7 and *S. enterica*. Based on these results, the use of CA in conjunction with HPP has good potential to serve as an alternative process for heat pasteurization of certain juices and meet the 5-log reduction performance standard as stipulated in the juice HACCP regulations.

Introduction

High pressure processing (HPP) is a relatively novel processing technique that has great potential in reducing microbial populations in foods without substantially altering nutritional and sensory characteristics. The application of HPP results in the killing of pathogenic and spoilage organisms to enhance microbial safety and shelf life of foods while creating minimal color change, limited flavor change, and mild texture change (Knorr, 1993; Cheftel, 1995). Pressures between 300 and 700 mega pascals (MPa), ~ 43,500 and 87,000 psi respectively, are capable of inactivating microorganisms while still maintaining fresh-like qualities of food products (Hoover et al., 1989; Stewart and Cole, 2001). With the ever growing consumer demand for nutritious, safe, wholesome foods, some sectors of the food industry have started to invest in alternative food processing technologies to thermal processing (Oey et al., 2008; Chen et al., 2015). Several pressurized fruit and vegetable juices and beverages have been produced in Europe, North America, Asia and Australia (Balasubramaniam et al., 2008) and are available in markets globally.

Over the past two decades, juices have been implicated in well-publicized foodborne disease outbreaks. Between 1995 and 2005, 21 outbreaks associated with juices were reported in the United States (Vojdani et al., 2008). The main pathogenic bacteria responsible for those outbreaks were *Escherichia coli* O157:H7, *Salmonella enterica* which were often associated with consumption of fresh apple juice, apple cider and orange juice (Bates el al., 2001; Besser et al., 1993; CDC, 1996; CDC, 1997; CDC, 1999; Cody et al., 1999; Cook et al., 1998; Parish, 1997). In recent years those same two pathogens were incriminated in outbreaks involving a variety of unpasteurized juices (Parish, 2009; Raybaudi-Massilia et al., 2009; CDC 2011; EFSA 2013, 2014, 2015). In an effort to reduce foodborne outbreaks and further strengthen food safety practices in the manufacturing of juice, legislation was passed requiring all juice processors to develop and

implement a Hazard Analysis Critical Control Point (HACCP) plan that includes a cumulative 5-log CFU/ml reduction (99.999%) performance standard for the pertinent pathogen (pathogen of public health concern) related to the juice being processed (US FDA, 2001). The U.S. FDA does not stipulate any type of process or antimicrobial intervention for use by processors to achieve that performance standard.

High pressure processing has the capability of achieving the performance standard without negatively changing nutritional and sensory characteristics of juices (Butz and Tauscher, 2002). Major economic issues related to use of HPP include very high initial investment cost for purchase and set up of equipment, high energy and maintenance costs, and shortened life of the equipment from repeated operation at very high pressures. Those economic issues justify research to improve the effectiveness of lower pressures and processing times to achieve a 5-log reduction of the pertinent pathogen in juices.

Following the hurdle technology approach proposed by Leistner and Gorris (1995) the potential for decreasing pressure levels and processing times for juices via use of antimicrobials in combination with HPP has been reported (Whitney et al, 2008; Espina et al., 2013; De Souza et al., 2016). An additional advantage of the hurdle approach is that additive or synergistic combinations can allow a reduction in level of each hurdle to achieve better preservation of nutritional and sensory characteristics of fresh foods (Espina et al., 2013). In this regard, reduction in level of individual hurdles can facilitate better food application of plant essential oils (EOs) and certain components of EOs which are potent antimicrobials but impart intense flavor changes to foods at concentrations required for antimicrobial effectiveness.

Cinnamaldehyde (CA) is a major component (70-95%) of cinnamon EO (Ross, 1976) and is generally recognized as safe (GRAS) by the U.S Food and Drug Administration and approved

for food use (21 CFR 182.60). Although CA used alone has exhibited antimicrobial activity against enteric pathogens in apple juice (Baskaran et al., 2010; Friedman et al. 2004; Loeffer et al., 2014), watermelon juice (Siddiqua et al., 2014; Jo et al., 2015) and carrot juice (Hernandez-Herrero et al, 2008), published reports on the use of CA in combination with HPP to inactivate enteric pathogens in juices are scarce. Accordingly, the objective of the present study was to investigate the effect of low concentrations of CA, when used in conjunction with HPP, on the reduction of *E. coli* O157:H7 or *S. enterica* in carrot juice and a mixed berry juice at refrigeration temperature (4°C).

Materials and Methods

Bacterial cultures and culture conditions. Five serotypes of *Salmonella enterica* (Enteritidis-ATCC13076, Heidelberg, Typhimurium-ATCC 14802, Gaminara-8324, and Oranienburg-9329), and five strains of *Escherichia coli* O157:H7 (FRIK125, ATCC 35150, ATCC 43894, ATCC 43895, and 93-062) from the culture collection of the Microbial Food Safety Laboratory, Iowa State University, Ames, IA used in the present study. Frozen (-70 ºC) stock cultures in brain heart infusion (BHI) broth (Difco,; Becton Dickinson, Sparks, Md) supplemented with 10% (vol/vol) glycerol were thawed under cold running water and activated in tryptic soy broth (TSB; Difco, Becton Dickinson) at pH 7.2 and incubated at 35 °C. At least two consecutive 18 to 24-h transfers of each stock culture were performed before using the cells for each experiment.

Preparation of inoculum. To prepare a 5-strain mixture of each pathogen, equal volumes (6 ml per culture) of *S. enterica* or *E. coli* O157:H7 working cultures were combined in a sterile centrifuge tube. The cells were harvested by centrifugation (10,000 x g, 10 min, 4 °C) using a Sorvall Super T21 centrifuge (American Laboratory Trading, Inc., East Lyme, CT) and

washed once in sterile 0.85% (wt/vol) saline. The pelleted cells were suspended in fresh 0.85% (wt/vol) saline to obtain a final viable cell concentration of ~10^9 CFU/ml. Viable colony counts of the washed cell suspensions were evaluated by serially diluting (10-fold) the suspensions and surface plating samples on tryptic soy agar (Difco, Becton Dickinson) supplemented with 0.6% yeast extract (TSAYE). The cell suspensions were used to inoculate BHI broth or fruit and vegetable juice blends.

Preparation and inoculation of juices. The CRJ and MBJ with no added preservatives were purchased from a local grocery store in Ames, IA. The CA was purchased from Sigma-Aldrich, Milwaukee, Wisconsin. Juices were stored in a walk-in cooler at 4 ^{0}C ± 1 ºC. The juices were vigorously shaken and 400 ml of each juice was aseptically transferred to sterile glass bottles. The bottles of juice were held at 4 ºC until filter-sterilized CA was added to them followed by inoculation with *E. coli* O157:H7 or *Salmonella*. The CA was added to each juice to obtain 0.10, 0.15, and 0.25 µl/ml. Juices without CA served as control. Bottles of juice were vigorously shaken and then inoculated with 4.0 ml of the suspension of the washed cells to give a final cell concentration of ~10^7 CFU/ml for each pathogen. After inoculation, each bottle of juice was swirled to mix its contents and stored at 4 ^{0}C in a walk-in cooler until they were exposed to HPP.

Measurement of pH and Brix. Measurements of pH and ºBrix were performed on non-inoculated juice on the day of inoculation. The pH measurements were taken using an Orion Model 525 pH meter (Orion Research, Inc., Boston, Massachusetts) fitted with a glass electrode. Before performing the pH measurements, all juice samples were tempered to 23 °C ± 1 °C. Brix measurements were performed using a digital Pocket Refractometer PAL (ATAGO, USA, Inc.).

Packaging of juice samples for high pressure treatment. Samples (5.0-ml) of inoculated juice were dispensed into separate sterile Type 402 polyester pouches (KAYPAK Corporation, Minneapolis, MN, USA). The pouches were 0.063 cm thick with an oxygen permeability of 118.65 cc/m^2/day (ASTM D-3985) and a carbon dioxide permeability of 845 cc/m^2/day at 23 ºC. Care was taken to seal the packaged juice samples with the minimum of headspace as practically possible. For safety, each sealed pouch of juice was placed in a secondary plastic bag, heat-sealed, then inserted in a third plastic bag containing 1.0 ml of sodium hypochlorite solution (100 ppm chlorine) and heat-sealed. Each triple-packaged juice sample was finally placed in a Cryovac plastic bag and vacuum-sealed in a Multivac A 300/51 vacuum packaging machine (Multivac, Sepp Hagenmueller, Gmblt and Co., Woltfertschwenden, Germany). All packages of juice were held in crushed ice until treated by high pressure. The total time from inoculation and packaging of juice samples to exposing them to high pressure was approximately 1.5 hours.

High pressure treatment. The packaged carrot juice and berry juice samples were subjected to high pressure treatment (400 and 300 MPa, respectively) in a FOOD-LAB 900 Plunger Press system (Stansted Fluid Power Ltd, Stansted, UK). The juice samples were pressurized for 60, 90, and 120 seconds. Propylene glycol was circulated at the desired temperature (~4 ºC) through the external jacket of the vessel and appropriately placed thermocouples allowed monitoring of temperature during pressurization of the samples. Average quasi-adiabatic temperature increases upon compression and the average rates of pressurization (MPa/ min) and depressurization were monitored.

Microbial analysis. Control juices (0 MPa) were analyzed for viable counts of pathogens at the start of pressure treatment of the other juice samples (~1.5 hours after inoculation). The pressure-treated juices were stored at 4 °C and analyzed for numbers of viable pathogens at day 0

(within 1 h after HPP), and at 1, 3, 7, 14, 28, 42, 56, and 75 days. Packages of juice were aseptically cut open using a sanitized scissors. One-ml samples of juice were serially diluted (10-fold) in buffered peptone water (BPW; pH 7.26) and surface plated on appropriate selective agar for each of the two pathogens tested. Sorbitol MacConkey agar (SMA) and xylose lysine Tergitol 4 (XLT) agar were used to enumerate *E. coli* O157:H7 and *S. enterica*, respectively. In order to count low numbers (less than 10 CFU/ml) of pathogens that might have survived the pressure treatment, 1.0-ml aliquots of juice were added to 2.0 ml samples of BPW to obtain 3-fold dilutions which were directly plated on appropriate selective agar. Numbers of survivors were reported as $\log_{10}$ CFU/ml. To cater for instances when there might be no bacterial colonies of the target pathogens on selective agar plates due to sub-lethally injured cells, 1.0-ml samples of juice were enriched using appropriate broth media and methods. The following is a description of the enrichment methods used for detecting viable pathogens at numbers lower than our detection limit (3 CFU/ml) and/or pathogens that were sub-lethally injured by the high pressure treatment.

Enrichment for *E. coli* O157:H7 was performed using the method described by Rodriguez et al. (2005) with slight modifications. Briefly, 1.0-ml samples of juice were each transferred to 9.0 ml of TSBYE with added selective cefexime-tellurite supplement in separate test tubes. The tubes of inoculated TSBYE were incubated at 42 ^{0}C for 6 h, followed by incubation at 35 ^{0}C for 48 h. After incubation samples of juice were streak-plated onto SMA and incubated at 37 ºC. After 24 hours of incubation the SMA plates were examined for bacterial colonies. Presumptive *E. coli* O157:H7 colonies were confirmed by testing for the presence of O157 and H7 antigens using the *E. coli* O157:H7 agglutination test (RIM®, *E. coli* O157:H7 Latex Test Kit, Thermo Fisher Scientific, Remel Prodts., Lenexa, KS).

Enrichment for *Salmonella* was performed as described by Hammack et al. (2001). In a primary enrichment step, 1.0-ml samples of juice were each added to separate tubes of UPB (9.0 ml per tube) and incubated at 35 ºC for 24 h. After incubation, a secondary enrichment was performed. This involved transferring 0.1-ml and 1.0-ml aliquots of UPB to 9.9-ml and 9.0-ml of tetrathionate broth respectively, followed by incubation at 35 ºC for 24 h. Additionally, 0.1-ml and 1.0-ml aliquots of UPB were transferred to 9.9-ml and 9.0-ml portions of Rappaport-Vassiladis (RV) broth respectively, followed by incubation at 42 ºC for 24 h. After incubation, the broth samples from secondary enrichment were streak-plated onto brilliant green agar and Hecktoen Enteric agar. The inoculated agar plates were incubated at 35 ºC for 24 hours before checking them for bacterial growth. Presumptive *Salmonella* colonies were aseptically picked and used to inoculate tubes of triple sugar iron (TSI) and lysine iron agar (LIA) slants. Colonies that gave biochemical reactions typical of *Salmonella* were confirmed via use of the OXOID *Salmonella* Latex Test Kit (Thermo Fisher Scientific, Remel Products, Lenexa, KS).

Statistical analysis. Three replications of each experiment were performed. Mean numbers of viable *S. enterica* and *E. coli* survivors were expressed as Log CFU/ml and statistically analyzed using SAS statistical software version 9.3 (SAS Institute Inc., Cary, N.C.). Treatment means were evaluated for statistically significant differences using the Waller-Duncan test. Significant differences were defined at $P < 0.05$ for all the experimental data

Results and Discussion

Background microflora, juice characteristics, and initial viable count. *Salmonella*, generic *E. coli* or *E. coli* O157:H7 was not detected in the CRJ or MBJ juice by direct plating or enrichment of non-inoculated juices without added CA. A low level of aerobic mesophilic background microflora in CRJ (5 ± 2 CFU/ml) and MBJ (0 to 3 CFU/ml) was detected based on

aerobic plate count of the non-inoculated juices. These results were expected because the juices were heat pasteurized and sold as refrigerated products. While pasteurization will destroy vegetative pathogens as well as substantial amounts of vegetative spoilage organisms, some thermoduric organisms and bacterial spores are likely to remain viable in the pasteurized product. The initial average pH of CRJ and MBJ was 6.25 and 3.59, respectively and initial °Brix values were 8.5 (for CRJ) and 12.3 (for MBJ). The addition of CA did not change in the pH or °BRIX in any of the two juices.

The average initial viable count of *S. enterica* or *E. coli* O157:H7 at the time of inoculation of control CRJ and CRJ with added CA was ~7.0 (± 0.2) $\log_{10}$ CFU/ml based on aerobic plate count of the cell suspension used to inoculate the juices. Numbers of viable pathogens (log CFU/ml) in CRJ just before HPP treatment ("pre HPP"; ~ 1.5 h after inoculation) averaged 6.48, 6.59 and 6.80 for CRJ with CA concentrations (µl/ml) of 0.25, 0.15, and 0 (control), respectively. These results reflect a slight loss of cell viability or sub-lethal injury inflicted by CA alone before the application of HPP. Viability of *E. coli* O157:H7 in CRJ following pressurization at 400 MPa is shown in Figures 1-3. Populations of *E. coli* O157:H7 in control CRJ (no added CA) were least affected by HPP at 400 MPa. Immediately after HPP (day 0) Log CFU/ml reduction of *E. coli* O157:H7 in control CRJ ranged from 1.35 (60 s of exposure) to 2.13 (120 s of exposure); log reductions in CA-treated cells (0.10, 0.15. and 0.25 µl/ml) ranged from 1.37 to 2.14, 1.58 to 2.59, and 2.26 to 3.21, respectively. In this regard none of the CA/HPP combinations achieved the 5-log reduction of *E. coli* O157:H7 in CRJ. Therefore, none of those CA/HPP treatments could serve as an alternative process for heat pasteurization and meet the 5-log reduction performance standard as stipulated in the juice HACCP regulations (US FDA, 2001)

Viability of the *E. coli* O157:H7 in control CRJ (no CA) declined slowly after HPP and after 75 days of refrigeration, viable counts of 3.35, 3.25 and 3.02 log CFU/ml were detected in controls that were pressurized (400 MPa) for 60, 90, or 120 s, respectively (Figures 1-3). Compared to control, loss of pathogen viability in pressure-treated juice containing CA increased rapidly with increasing CA concentration. At 3 days after HPP, complete elimination (negative enrichment) of viable *E. coli* O157:H7 in CRJ (with CA at 0.25 µl/ml) that was pressurized (400 MPa) for 90 or 120 seconds (Figures 2 and 3) was observed. At day 3, while no *E. coli* O157:H7 colonies were recovered on SMA, none was detected by enrichment tests even after 24 h and 48 h of incubation at 35 °C. These results indicate that CA increases the sensitivity of *E. coli* O15:H7 to high pressure at 400 MPa.

The inactivation of *S. enterica* by HPP of CRJ with or without CA is shown in Figures 4-6. Similar to our observations with *E. coli* O157:H7, as the pressure exposure times and CA levels increase, the numbers of salmonellae survivors decrease. A major observation is that *S. enterica* was more sensitive than *E. coli* O157:H7 to the CA/HPP combinations tested. At a CA concentration of 0.25 µl/ml used in conjunction with 400 MPa, *Salmonella* was reduced by more than 5.5-$\log_{10}$ CFU/ml at day 0 (~1 h after pressure treatment). At that time no salmonellae could be detected neither by plating nor enrichment of CRJ samples following pressurization at 400 MPa for 120 s. Based on enrichment tests this reduction was maintained through the 75-day storage of CRJ at 4 ºC.

The control MBJ (pH 3.59, 12.3 °Brix) reduced the initial viable counts (log CFU/ml) of each of the two pathogens from 7.0 to about 5.32 (a 1.68 log CFU/ml reduction) before the juice samples were pressurized (Figures 7 and 8). Prior to HPP reductions in viable counts for both pathogens in MRJ with added CA (0.1 or 0.15 µl/ml) were also observed; however, compared to

control, those reductions were not significant (P>0.05). Both pathogens exhibited a greater sensitivity to HPP in MBJ with or without added CA compared to their pressure sensitivity in CRJ. Log CFU/ml reductions of viable *E. coli* O157:H7 just after HPP (300 MPa, 60 s) were 1.35, 1.37, and 1.58 in CRJ with 0 (control), 0.10, and 0.15 µl/ml CA, respectively. At those same CA concentrations pressure conditions, log reductions of the pathogen were 2.24, 3.41 and 3.72, respectively. In this context log reductions were derived by calculating the difference in viable counts of the pathogen just before and soon after pressure treatment. A similar trend was observed with *S. enterica* in MBJ; log reductions after pressurization (300 MPa, 60 s) in MBJ were higher, 2.54 (control; 0 µl/ml CA), 4.52 (0.10 µl/ml CA) and 5.11 (0.15 µl/ml CA), compared to reductions in CRJ, 1.12 (control; 0 µl/ml CA), 3.20 (0.10 µl/ml CA) and 3.75 (0.15 µl/ml CA). MBJ samples with CA at 0.15 µl/ml and pressurized (300 MPa) for 90 or 120 s resulted in greater than a 5-$\log_{10}$ CFU/ml reduction of both pathogens based on selective plating immediately after pressure treatment; however, complete inactivation (negative enrichment) of the pathogens was observed only in pressure-treated (300 MPa, 120 s) MRJ with CA at 0.15 µl/ml (Data not shown).

A 5-$\log_{10}$ CFU/ml reduction of both *E. coli* O157:H7 and *Salmonella* was accomplished by high pressure processing combined with CA in both juices; however, in many instances a 5-$\log_{10}$ CFU/ml reduction did not occur instantaneously. To achieve a 5-$\log_{10}$ CFU/ml reduction of bacterial pathogens in carrot juice, increased pressure (>400 MPa) or increased concentration of CA (>0.25 µl/ml) is required. In contrast, for the more acidic MBJ, a lower pressure (300 MPa) in conjunction with CA (0.15 µl/ml) can be used to eliminate bacterial pathogens. The results of the present study demonstrate the potential of HPP and CA as an alternative processing strategy to thermal treatment for inactivating pathogens such as *E. coli* O157:H7 and *S. enterica* in juices. The properties of juices such as acidity, ºBrix, and phenolic content can influence the effect of the

combination treatments; therefore, validation of HPP in combination with CA is necessary for each different type of juice.

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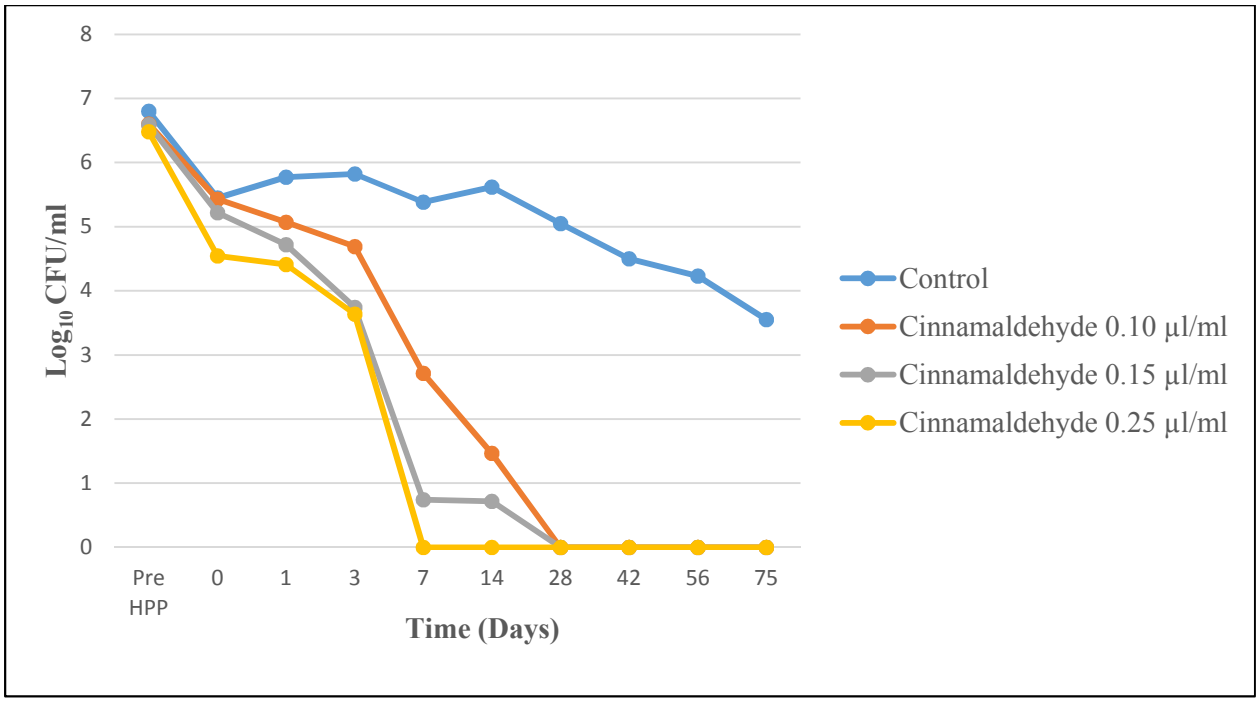

Figure 1. Effect of cinnamaldehyde and HPP (400 MPa, 60 seconds) against *E. coli* O157:H7 in carrot juice. Data represent means of three replications.

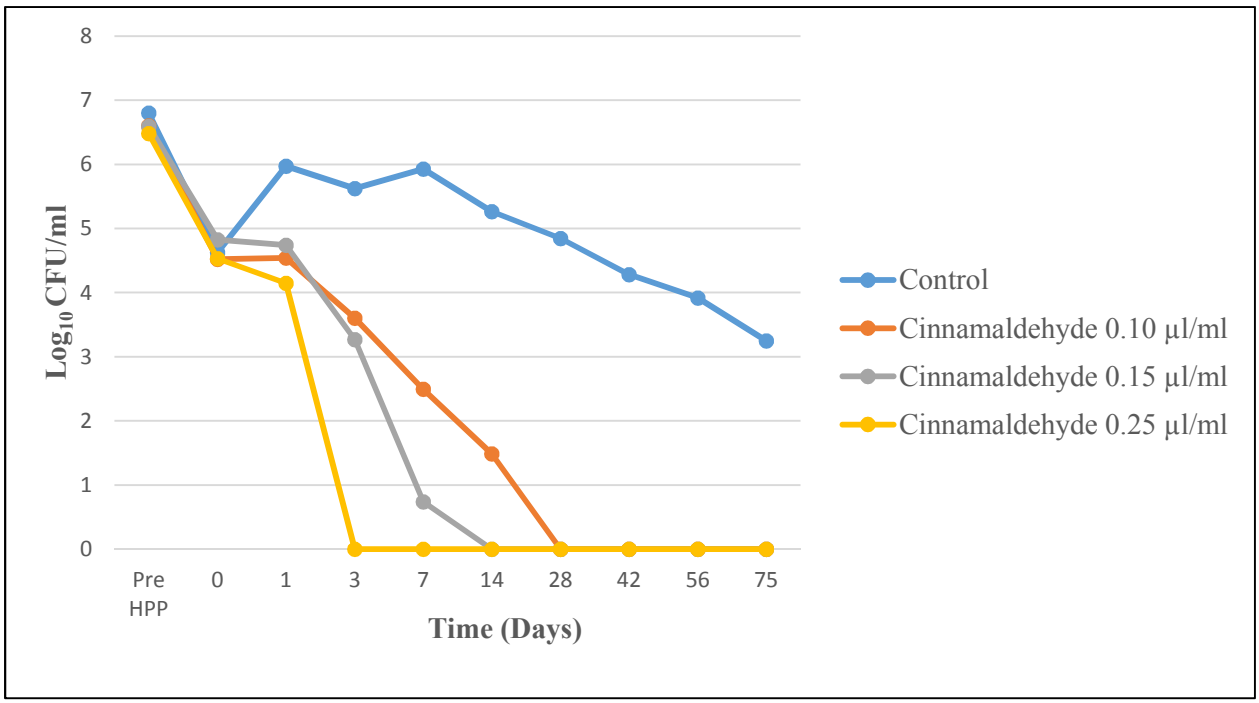

Figure 2. Effect of cinnamaldehyde and HPP (400 MPa, 90 seconds) on numbers of viable *E. coli* O157:H7 in carrot juice. Data represent means of three replications.

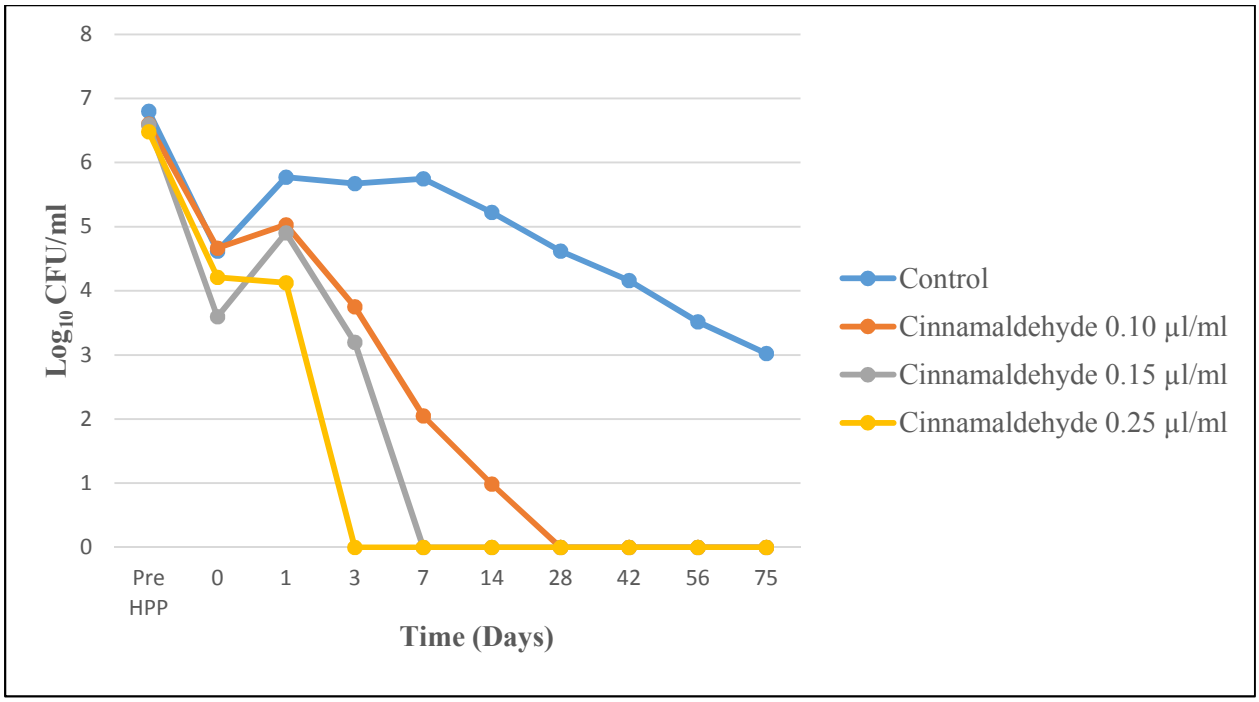

Figure 3. Effect of cinnamaldehyde and HPP (400 MPa, 120 seconds) on numbers of viable *E. coli* O157:H7 in carrot juice. Data represents means of three replications.

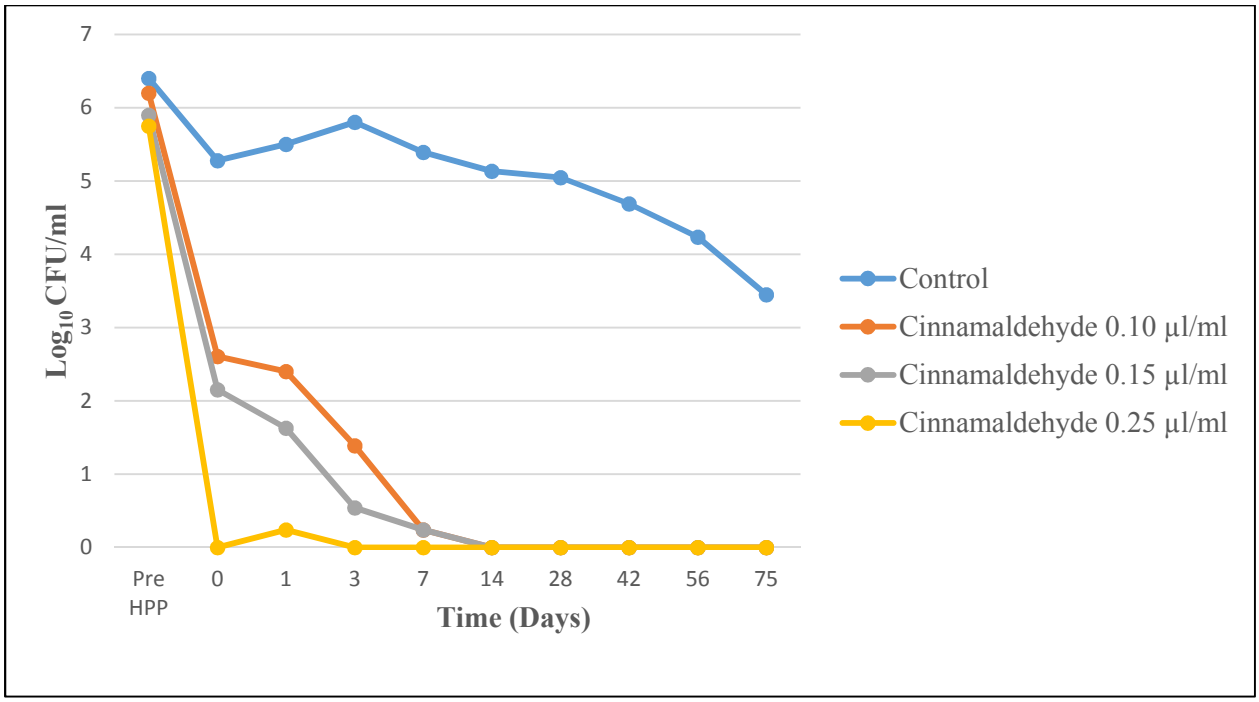

Figure 4. Effect of cinnamaldehyde and HPP (400 MPa, 60 seconds) on numbers of viable *Salmonella* in carrot juice. Data represents means of three replications.

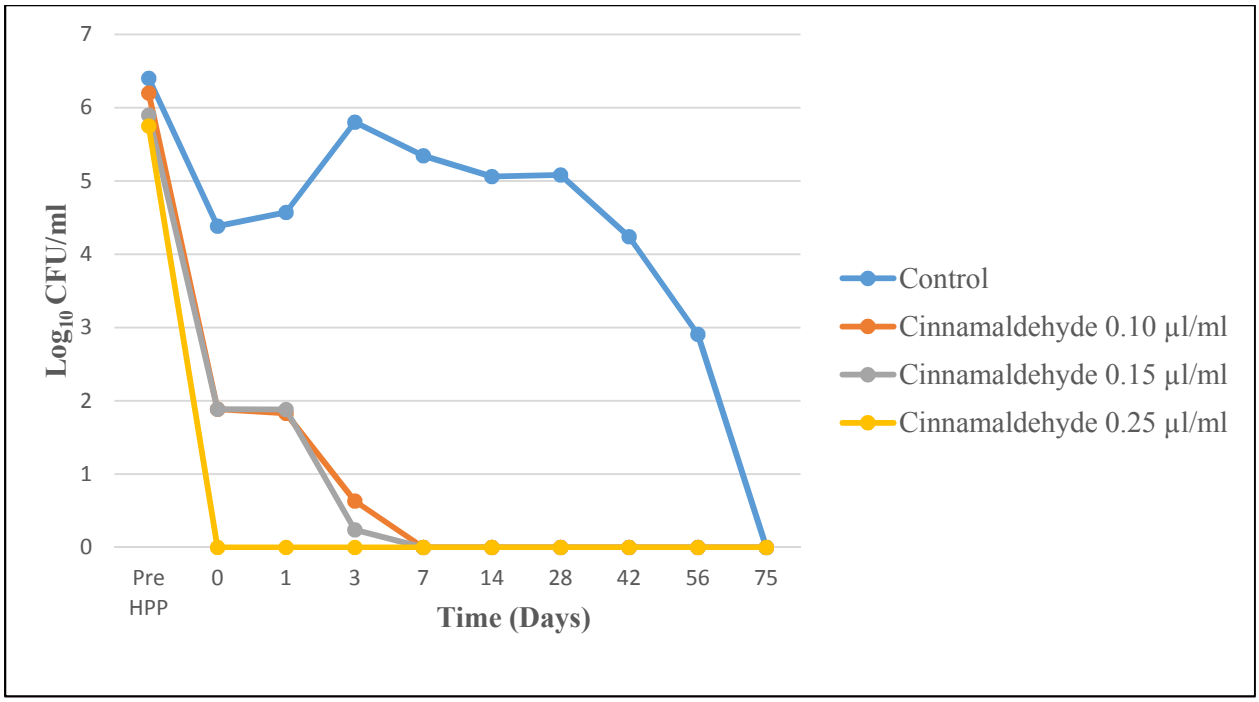

Figure 5. Effect of cinnamaldehyde and HPP (400 MPa, 90 seconds) on numbers of viable *Salmonella* in carrot juice. Data represent means of three replications.

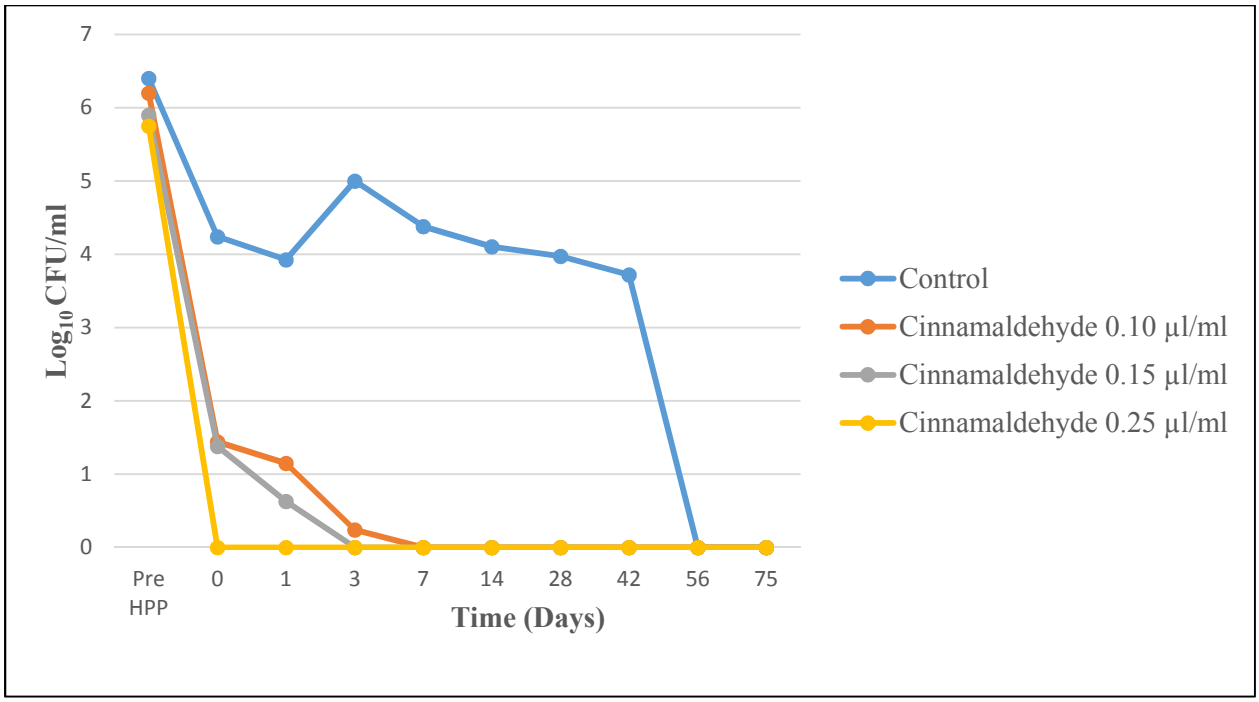

Figure 6. Figure 4. Effect of cinnamaldehyde and HPP (400 MPa, 120 seconds) on the numbers of viable *Salmonella* in carrot juice. Data represent means of three replications.

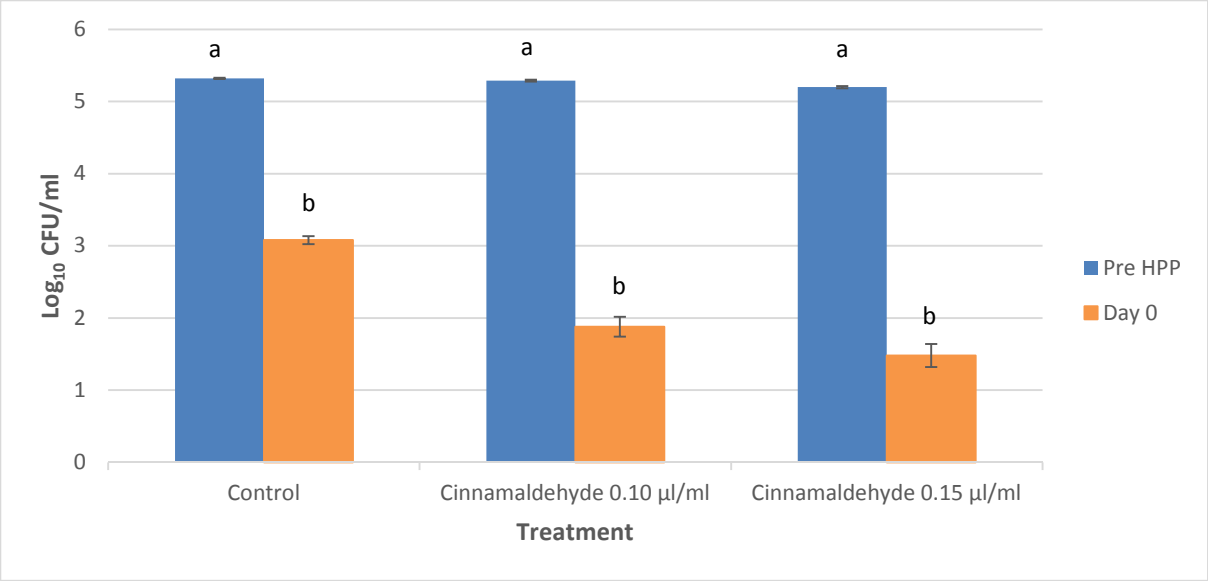

Figure 7. Effect of cinnamaldehyde and HPP (300 MPa, 60 seconds) on numbers of viable *E. coli* O157:H7 in mixed berry juice. Data represent means of three replications. Bars within one treatment with different letters are significantly different (P <0.05).

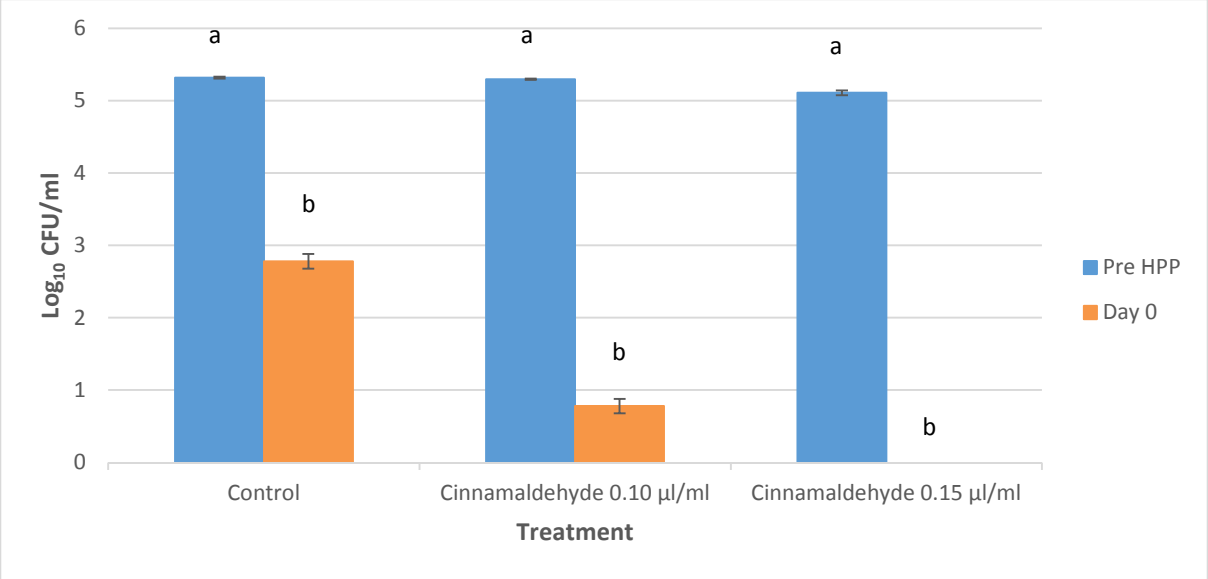

Figure 8. Effect of cinnamaldehyde and HPP (300 MPa, 60 seconds) on numbers of viable *Salmonella* in mixed berry juice. Data represent means of three replications. Bars within one treatment with different letters are significantly different (P <0.05).

CHAPTER 6.

BACTERICIDAL ACTION OF CINNAMALDEHYDE AGAINST *ESCHERICHIA COLI* O157:H7 CORRELATES WITH PERMEABLIZATION OF THE CYTOPLASMIC MEMBRANE

A paper to be submitted to the Journal of Food Protection

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Key words:, foodborne pathogens, cinnamaldehyde, leakage, cytoplasmic membrane

Abstract

Cinnamaldehyde (CA), a flavor component of cinnamon spice, exhibits activity against Gram-negative and Gram-positive bacteria; however, its mechanism of bactericidal action is inconclusive. Physiological saline (0.85% w/v NaCl) alone (control) or with added CA at 2.2, 2.5, and 2.8 µl/ml, was inoculated with washed cells of *Escherichia coli* O157:H7 (FRIK 125) to obtain a final cell concentration of ~ 7.89 $\log_{10}$ CFU/ml. At 0, 5, 10, and 15 minutes, cell suspensions were sterilely filtered and each filtrate was analyzed for absorbance at 260 nm. Cell viability was evaluated by plating cell suspension on both non-selective and selective agar media and counting bacterial colonies after incubation (35 °C) for 48 hours. Absorbance ($A_{260\ nm}$) values increased substantially with increasing CA concentrations which simultaneously produced a dramatic decline in viability of *E. coli*. At 5, 10, and 15 min of exposure to CA (2.8 µl/ml) initial numbers of viable *E. coli* decreased by and 1.65, 4.77, and 6.9 $\log_{10}$ CFU/ml, respectively, based on colony counts on non-selective agar media. At those same time intervals CA-treated cell suspensions produced no bacterial colonies on selective agar media indicating that survivors were sub-lethally injured. A strong correlation was observed between initial release rate of cellular material with $A_{260\ nm}$ and the death rate of *E. coli* (R^2 = 0.939). Based on these results we conclude that permeabilizaton of the cytoplasmic membrane plays a major role in the bactericidal action of CA.

Introduction

Historically spices and herbs have been used to either enhance the aroma and flavor of foods or to extend the shelf life of packaged food products (Beuchat, 1994). The increasing food industry trend in use of compounds from spices as antimicrobial food additives started in the 1980s with negative consumer perceptions of traditionally used food preservatives such as sorbates, benzoates and nitrates (Shelef, 1983). Additionally, with the growing consumer demand to

eliminate the use of synthetic food additives, food manufacturers have started to explore the use of naturally derived antimicrobial compounds (Burt, 2004).

The antimicrobial activity of several spice extracts is mainly attributable to substituted aromatic molecules such as carvacrol, eugenol and cinnamaldehyde (CA) (Jay and Rivers, 1984; Juven et al., 1984; Moleyar and Narasimham, 1992; Hyldgaard et al., 2012). There is much interest in use of CA as an antimicrobial agent in foods because of its proven activity against both Gram-negative and Gram-positive foodborne bacteria (Bowles and Miller, 1993; Bowles et al., 1995; Helander et al., 1998). However, the application of naturally derived antimicrobials in food products has progressed slowly. This slow progression is due to factors such as difficulty in identifying active components in extracts from natural sources, cost of the active component, and use of high concentrations that negatively alter sensory quality of foods (White et al., 2011**;** Nychas and Skandamis, 2003**;** Roller and Board, 2003).

The problem of using high concentrations of spice extracts to achieve desired antimicrobial effects in foods may be circumvented via the hurdle technology concept through which the extract is used as a part of a multi-component antimicrobial system (White et al., 2011; Adams and Smid, 2003; Blaszyk and Holley, 1998). Development of effective multicomponent antimicrobial systems for foods requires a good understanding of the mode of action of each specific antimicrobial (Gill and Holley, 2004).

As a phenylpropene isolated from the essential oil of cinnamon, CA is typically characterized as a pale yellow and viscous liquid. When extracted from cinnamon, CA is commonly referred to as trans-cinnamaldehyde which contains a phenyl group attached to an unsaturated aldehyde. Aldehyde groups are reactive and cross-link with DNA and proteins through amine groups, leading to interference with normal cellular functions (Feron et al., 1991). Although

the antimicrobial effects of several spice extracts and their components are usually attributed to alterations of the cell membrane, the antibacterial mode of action of CA is inconclusive. Cinnamaldehyde is believed to inactivate enzymes associated with cytokinesis and inhibit ATPase at sub-lethal concentrations, and disruption the cell membrane at lethal concentrations (Hyldgaard et al., 2012). At sub-lethal concentrations, CA inhibited activity of transmembrane ATPase in *E. coli* (Helander et al., 1998). Based on that finding, it was concluded that CA traverses the outer membrane, enters the periplasm and possibly contacts the cytoplasmic membrane (Helander et al., 1998). Using isolated cell membranes Gill and Holley (2006) demonstrated decreases in ATPase activity with increasing concentrations of CA in the range of 13.6 to 1362 µg/ml. However, those same authors suggested that ATPase inhibition was not the primary cause of death in *E. coli* because CA concentrations (681 – 1362 ug/ml) which inhibited ATPase activity also produced membrane disruption in that organism.

While there are some postulations on the antimicrobial mode of action of CA, there is a scarcity of published reports that specifically correlate loss of microbial viability with CA-induced cellular lesions in bacteria. Accordingly, the main objective of the present study was to determine if the bactericidal action of CA against *E. coli* O157:H7 (FRIK 125) may be attributed to cellular loss of cytoplasmic components.

MATERIALS AND METHODS

Bacterial cultures and culture conditions. *Escherichia coli* O157:H7 (FRIK 125) was used in the present study. The culture was earlier obtained from Dr. Charles Kaspar, Department of Bacteriology, University of Wisconsin – Madison, and maintained in the culture collection of the Microbial Food Safety Laboratory, Iowa State University, Ames, IA. The Stock culture was kept frozen (-70 ºC) in Brain heart infusion (BHI) broth (Difco, Becton Dickinson, Sparks, MD)

supplemented with 10% (vol/vol) glycerol. The stock culture was activated in BHI broth (pH 7.4) and incubated at 35 °C. At least two consecutive 18 to 24-h transfers of each stock culture were carried out prior to using the cells in each experiment.

Determination of bactericidal concentrations of cinnamaldehyde. The minimum bactericidal concentration (MBC) of CA for *E. coli* O157:H7 was evaluated using a standard broth dilution assay as described by Conte and Barriere (1992). Briefly, serial dilutions (2-fold) of CA in BHI were aseptically prepared to obtain CA concentrations ranging from 12 µl/ml to 0.187 µl/ml. Each tube of BHI with CA was inoculated with *E. coli* O157:H7 to give ~5.7 Log CFU/ml and incubated at 35 °C for 24 h. Numbers of survivors of the pathogen were determined by plating aliquots of diluted BHI + CA on tryptic soy agar (Difco) supplemented with 0.6% yeast extract (TSAYE) and counting bacterial colonies after 48 h of incubation (35 °C). The lowest concentration of antimicrobial that resulted in $\geq$ 99.9% ($\geq$3 Log) kill within 24 h was identified as the MBC (NCCLS, 2002).

Preparation of treatments. A commercial preparation of CA (Aldrich C80687) was purchased from Sigma-Aldrich, St. Louis, MO. Treatment solutions of CA (2.2 µl/ml, 2.5 µl/ml, and 2.8 µl/ml based on a 100-ml volume) were prepared in 99 ml of 0.85% (w/v) NaCl (saline). Each treatment (99 ml) was sterilely filtered using disposable Nalgene Type S Sterilization Filter Units (0.45 µm pore size) (Curtin Matheson Scientific, Inc, Broadview Heights, OH), aseptically transferred into separate, sterile bottles and held at ambient temperature (23 ± 1 °C) prior to inoculation. Saline without added CA served as control.

Preparation of bacterial cell suspension. The *E. coli* O157:H7 was cultured aerobically in 60 ml of brain heart infusion broth (BHI) at 35 °C. After 24 h of incubation, the cells were be

harvested by centrifugation in a Beckman J2-21 centrifuge (Beckman Instruments Inc., Palo Alto Calif.) operating at 10,000 x g for 10 min at 4 °C. The pelleted cells were suspended in fresh 0.85% (w/v) saline (60 ml) and then harvested again by centrifugation as previously described. The pelleted washed cells were suspended in fresh 0.85% (w/v) saline (6 ml) to give a final viable cell concentration of ~ 10 Log_{10} CFU/ml as determined by plate counts on TSAYE.

Exposure to cinnamaldehyde treatment. The control and each solution of CA (99 ml) was inoculated with 1.0 ml of cell suspension to give a final concentration of ~ 7.89 Log_{10} CFU/ml. Cells were mixed immediately by vortexing and then held at ambient temperature. Approximately 14 s elapsed from the time of inoculation to the time cells were diluted in buffered peptone water (BPW; Becton, Dickinson) for subsequent surface-plating on agar media. Therefore, time point zero represents ~ 14 s of exposure of the cells to CA treatment. Aliquots (5 ml) of treated cell suspension were removed from the treatment tubes at time 0, 5, 10, and 15 minutes. One ml was added to 9 ml BPW, mixed by vortexing, and then plated onto selective agar namely, Sorbitol Mac Konkey agar (SMA: Becton, Dickinson) supplemented with potassium tellurite and cefixime (TC-SMA) and non-selective agar (TSAYE). The remaining 4.0 ml of treated cell suspension was immediately filtered through a 25 mm, 0.45 µm pore size Luer Lock syringe filter attached to a Luer Lock plastic syringe (Curtin Matheson Scientific).

Cell viability. Viability of cells was determined by serially diluting treated cells in BPW followed by surface plating 0.1 or 1.0 ml onto TSAYE and Sorbitol MacKonkey agar (SMA). The inoculated agar plates were incubated at 35 °C and bacterial colonies were counts at 48 h.

Cellular leakage of 260 nm absorbing material. Cell filtrates were examined spectrophotometrically for 260 nm absorbing material using a genesis 10S UV-VIS

Spectrophotometer, Model G10S UV-Vis (Thermo Fisher Scientific, Hudson, NH). Quartz cuvettes containing approximately 3.5 ml of cell filtrate were scanned at a wavelength of 260 nm. Separate cuvettes containing saline with added CA (at all concentrations of CA tested) were also examined at 260 nm to determine any contribution of CA to absorbance values obtained in the experiments. All trials were replicated 3 times.

Cellular leakage of lactate dehydrogenase. To determine if cells exposed to CA released lactate dehydrogenase (LDH) in the suspending medium, sterile cell-free filtrates were prepared (as previously described) from *E. coli* O157:H7 cell suspensions of control (no CA) and CA-treated cells. The Pierce™ LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific, Waltham, MA) was used to spectrophotometrically demonstrate LDH activity in the filtrates. A vial of lyophilized substrate mix was added to 11.4 ml of ultrapure water in a 15 ml conical tube. The contents of the tubes were gently mixed to dissolve the lyophilized substrate mix. Frozen assay buffer (0.6 ml) was thawed under running tap water and then added to the tube containing the substrate mix. Tubes of reaction mixture were gently inverted several times and covered with foil to protect them from light until they were used (within 15 minutes of preparation). Aliquots (0.5-ml) of filtrate from control and CA-treated cell suspensions were placed in separate quartz cuvettes followed by the addition of 0.5 ml of reaction mixture to each cuvette. Positive and negative controls for the enzyme activity assay were also prepared. All cuvettes of reaction mixtures with or without added filtrate were incubated at 23 ± 1 °C for 30 minutes. Following incubation 0.5 ml of stop solution was added to each cuvette. All samples were measured spectrophotometrically (490 nm) using a genesis 10S UV-VIS Spectrophotometer, Model G10S UV-Vis.

Transmission electron microscopy. Control and CA-treated *E. coli* O157:H7 cells in 0.85% (w/v) saline at 23 ± 1 ºC were harvested by centrifugation (10,000 x g, 10 min, 4 ºC).

Aliquots (3-µl each) of cells in saline were placed onto a carbon film coated copper grid for 1 minute. The supernatant was removed by wicking from the side with a piece of filter paper and 3µl of 2% aqueous uranyl acetate was placed onto the grid for 30 seconds. The stain was removed by wicking and the grid was allowed to dry. Images were made using a JEOL 2100 scanning transmission electron microscope at an accelerating voltage of 200 kV (Japan Electron Optics Laboratory, USA, Peabody, MA).

Statistical Analysis. Three replications of each experiment were performed. Mean numbers of viable *Escherichia coli* O157:H7 survivors were statistically analyzed using SAS statistical software version 9.3 (SAS Institute Inc., Cary, N.C.). Treatment means were evaluated for statistically significant differences using the Waller-Duncan test. Significant differences were defined at $P < 0.05$ for all the experimental data.

RESULTS AND DISCUSSION

The MBC is defined as the lowest concentration of antimicrobial that results in $\geq$ 99.9% ($\geq$3 Log) kill of the target organism within 24 h (NCCLS, 2002). The MBC of CA for *E. coli* O157:H7 was 1.5 µl/ml; however, that concentration of CA, while satisfying the definition of MBC, had very little to no effect on viability of the pathogen within 15-min of exposure of the pathogen to CA (data not shown). Therefore, higher CA concentrations that exhibited a killing effect within 15 minutes were selected for evaluation. For each of the three replicate experiments performed, the viability of *E. coli* O157:H7 in control saline remained relatively constant for the entire test period (15 min). As expected, the use of CA concentrations higher than the MBC resulted in loss of *E. coli* viability in a shorter time. As the time of exposure to CA increased the viability of the pathogen decreased. For example, after 5, 10, and 15 min of exposure to CA (2.8

µl/ml) initial numbers of viable *E. coli* decreased by and 1.65, 4.77, and 6.9 $\log_{10}$ CFU/ml, respectively, based on colony counts on TSAYE (Figure 1).

The previously stated results are in agreement with those of previous studies that reported potent bactericidal action of CA (Kim et al., 2004; Ali et al., 2005). Our results demonstrate that above its MBC for *E. coli* O157:H7, CA exhibits a dose-dependent bactericidal action on stationary-phase cells of the pathogen during 15 min of exposure. With regard to the antimicrobial effects of CA concentrations, three important conceptions have emerged from previous research: i) CA at low concentrations possibly inhibits enzymes used in cytokinesis (Kwon et al., 2003) or less crucial cellular functions, ii) at greater but sub-lethal concentrations CA inhibits ATPase activity (Gill and Holley, 2006 a,b) and iii) at bactericidal concentrations, CA destabilizes the cell membrane (Gill and Holley, 2006a). Based on this information it is likely that CA, depending on its concentration, may inflict multiple injuries in bacteria thus making it challenging to determine its precise antimicrobial mode of action.

Several studies have reported that CA interacts with the bacterial cell membrane; however, the precise mode of CA's interaction to cause membrane destabilization is unclear (Hyldgaard et al., 2012). Exposure of *E. coli*, *Salmonella enterica*, *Pseudomonas fluorescens*, and *Brocothrix thermosphacta* to CA resulted in alteration of the membrane lipid profile of these organisms to give a more rigid membrane, a possible compensatory effect for the fluidizing action of CA (Di Pasqua et al., 2006; 2007). Di Pasqua et al. (2007) demonstrated that CA disintegrated the cell envelope of *Staphylococcus aureus* and caused structural alteration of the outer membrane in *E. coli* O157:H7.

The intact bacterial cytoplasmic membrane will generally prevent loss of material from the cytoplasm; therefore, cellular leakage of cytoplasmic constituents is a fundamental indication the

intact structure of the cytoplasmic membrane has been compromised (Denyer and Hugo, 1991). The lactate dehydrogenase (LDH) assay was consistently negative for all concentrations of CA tested in the present study. These results are conclusive because the positive control indicated that the assay efficiently detected the LDH. This result was surprising because we could consistently detect the occurrence of 260 nm absorbing material in the filtrate of CA-treated cells but not in the filtrate of control cells (no CA treatment). It is likely that cytoplasmic membrane damage by CA causes small pores that might preclude passage of large proteins but allow smaller molecules to leak out of the cell. LDH being a protein and being larger than amino acids of purines and pyrimidines might not pass through very minute openings. Also, LDH might have leaked out of the cell but immediately encountered CA in the extra cellular environment and became inactive. This assumption is based on published reports that highlight the inhibitory action of CA against several enzyme systems (Gill and Holley, 2006b; Tanaka et al., 2011).

Results of the present study show no leakage of 260 nm absorbing material (AM) could be detected in filtrate from control cell suspensions. At CA concentrations of 2.2, 2.5, and 2.8 µl/ml, leakage of 260 nm absorbing material (AM) from *E. coli* O157:H7 increased with time (Table 1). Our results are in agreement with those of Shen et al (2015) who reported that exposure of *E. coli* and *S. aureus* to CA (0.31 mg/ml) resulted in several abnormalities including membrane damage and cellular leakage of 260 nm-absorbing material. Evaluation of our data on cell leakage and viability, as affected by CA, revealed a strong correlation ($R^2 = 0.939$) between cellular leakage (initial change in 260 nm AM per min) and death rate (Log reductions per min) (Figure 2). Based on the results of the present study, more specifically the correlation ($R^2=0.939$) between leakage of 260nm AM from CA-treated cells and their death rate, we conclude that CA-induced death of *E. coli* O157:H7 involves the permeabilization of the cytoplasmic membrane.

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TABLE 1. Effect of varying concentrations of cinnamaldehyde on DNA leakage of *Escherichia coli* 0157:H7 in 0.85% saline solution for 15 minutes.

	260 nm AM[x]			
Treatment (µl/ml)	0.23m	5m	10m	15m
Control	0.001 ± 0.000a	0.001 ± 0.000a	0.001 ± 0.000a	0.001 ± 0.000a
Cinnamaldehyde (2.2)	0.007 ± 0.008b	0.009 ± 0.009b	0.011 ± 0.006b	0.015 ± 0.004b
Cinnamaldehyde (2.5)	0.008 ± 0.004b	0.011 ± 0.008b	0.013 ± 0.007b	0.018 ± 0.006b
Cinnamaldehyde (2.8)	0.003 ± 0.006b	0.012 ± 0.002b	0.020 ± 0.002c	0.021 ± 0.003c

[x]Each reported value for viable count represents the mean (standard deviation) of three independent replications of the experiment

[a,b,c]Means with a different letter within a column differ significantly ($P<0.05$)

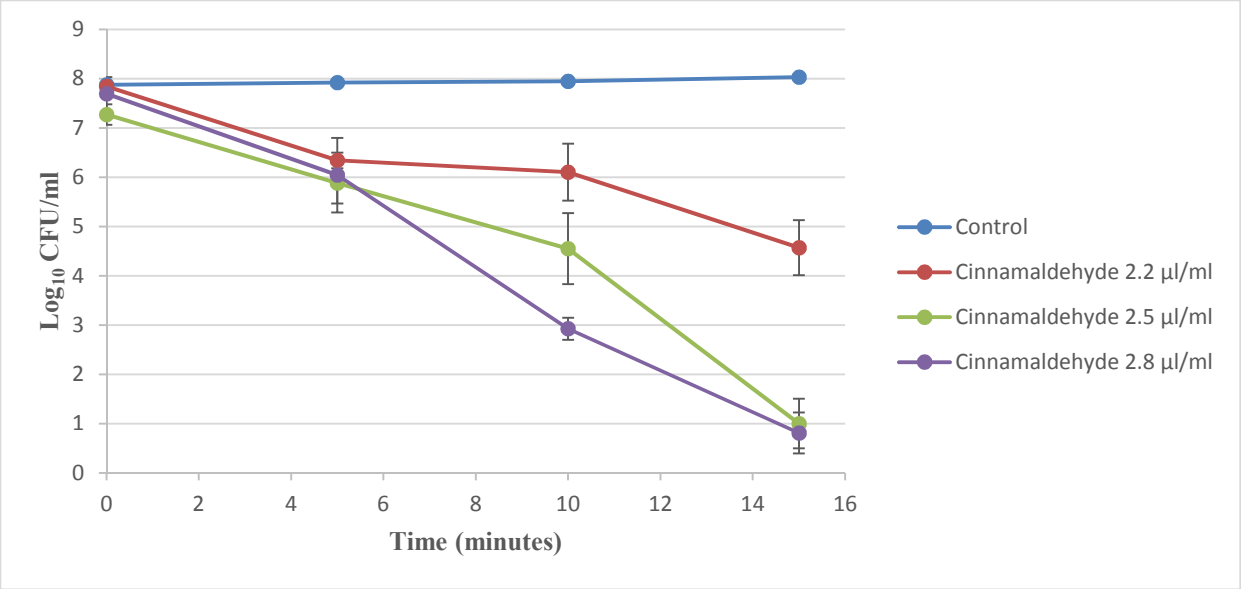

Figure 1. ***E. coli*** **O157:H7 survivors plated on nonselective media after exposure to cinnamaldehyde in 0.85% saline[x].**

[X]Each reported value for viable count represents the mean (standard deviation) of three independent replications of the experiment.

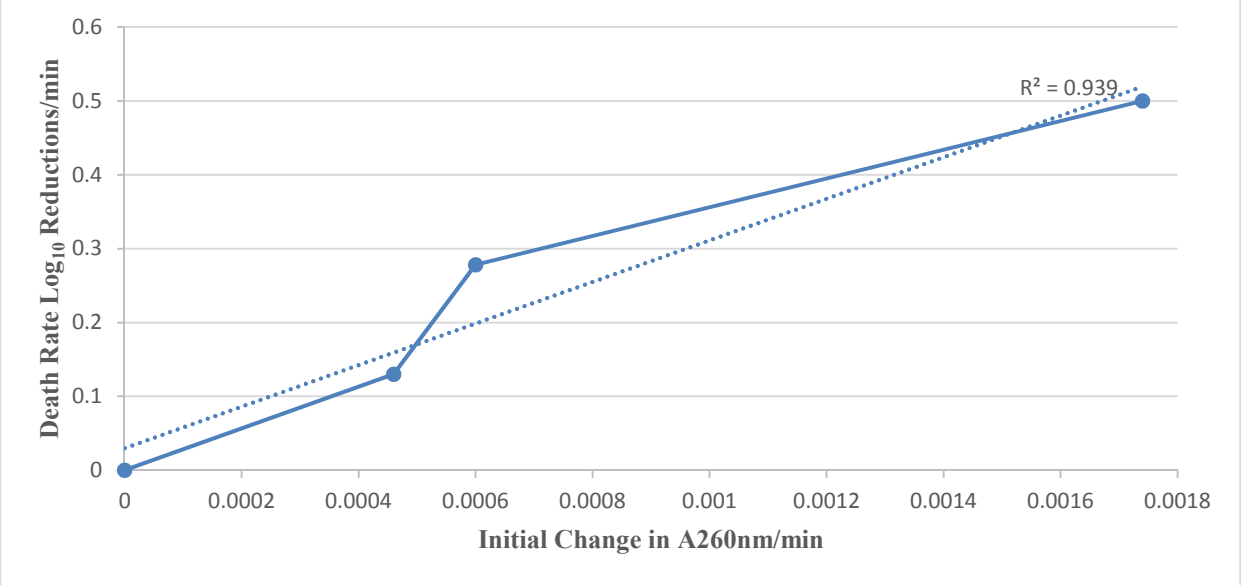

FIGURE 2. Relationship between initial rate of leakage of 260 nm AM and death rate for *E. coli* O157:H7, exposed to 2.2, 2.5 and 2.8 µl/ml cinnamaldehyde in 0.85% saline.

CHAPTER 7.

GENERAL CONCLUSIONS

Considering the growing consumer demand for alternatives to thermal treatments which can alter nutritional and sensory properties of foods, the application of non-thermal techniques for ensuring microbial safety of foods will become more widespread. Based on the results of experiments reported in the present thesis two components of plant essential oil components namely, cinnamaldehyde (CA) and geraniol (GER) are strongly bactericidal depending on the concentration used. Both naturally derived antimicrobials kill foodborne pathogens in a dose-dependent manner. The pathogens tested in these studies (*Escherichia coli* O157:H7 and *Salmonella enterica*) are more sensitive to the lethal effects of CA or GER in a more acidic juice such as mixed berry juice compared to a less acidic juice such as carrot juice.

The addition of CA to juices sensitize *Escherichia coli* O157:H7 and *Salmonella enterica* to the antimicrobial effects of high pressure processing and therefore permits the use of lower pressures to destroy pathogens in juices. To date the bactericidal mechanism of bactericidal action of CA is yet to be clarified. Based on these results of the present studies we conclude that permeabilizaton of the cytoplasmic membrane plays a major role in the bactericidal action of CA. It is concluded that CA and GER are effective antimicrobials from natural sources for inactivating bacterial pathogens in fruit and vegetable juices to enhance the microbial safety of these nutritious food products. Also, the use of CA in conjunction with HPP has good potential as an alternative process for thermal treatment of juices to comply with the 5-log reduction performance standard as stipulated in the juice HACCP regulations. Addition of CA to juices increased the sensitivity of the pathogens to HPP with S. enterica exhibiting a greater loss in viability than E. coli O157:H7 to the CA/HPP combinations tested.